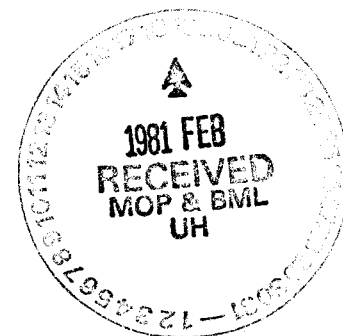


MARINE SKILL REPORT SUBMITTED TO THE
UNIVERSITY OF HAWAII MARINE OPTION PROGRAM
An Internship With Brewer Chemical Corporation
Training in Analytical Techniques for
Water Quality Chemistry



DURATION

September 1, 1980 - December 12, 1980

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REPORT DATE

January 17, 1981

ABSTRACT

During the fall of 1980 I completed a single semester internship in the analytical laboratories of the Brewer Chemical Corporation at Iwilei, Honolulu. The internship focused upon training in analytical techniques for water quality chemistry and related subjects. This report details the internship and, specifically, what I gained from it.

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INTRODUCTION

I am presently majoring in chemistry at the University of Hawaii - Manoa, and plan to concentrate my studies in the area of marine analytical chemistry. Through the University of Hawaii Marine Option Program, I was able to engage in an internship provided by the Brewer Chemical Corporation between September 1st and December 12th, 1980. I was trained in analytical techniques for water quality chemistry, and in other areas of analytical chemistry in the analytical laboratories of the Brewer Chemical Corporation plant at Iwilei. Here then is a detailed report of the internship.

THE TRAINING SITUATION

For the duration of the internship, I worked for ten hours each week in the water quality laboratory of the Brewer Chemical Corporation at Iwilei, under the supervision of laboratory personnel. All materials and equipment used were supplied by the Brewer Chemical Corporation.

The analytical laboratory in which I worked performs various analyses for private companies and organizations, as well as some state and federal agencies; this is one of the many commercial operations of the Brewer Chemical Corporation. The types of analyses performed are many and varied, and I mainly concentrated on analyses and techniques that had some sort of relevance to marine science, or at least involved the analysis or use of water.

I received direct training from several of the laboratory staff. Generally, a member of the laboratory staff would explain a specific analysis or technique and demonstrate the process in the laboratory, making sure that I was able to understand and perform the process. I also had access to literature detailing the analyses.

When laboratory staff were occupied, I would consult the laboratory supervisor to determine analyses that needed to be done; I could then

independently perform such analyses if I had previously dealt with them, thereby increasing my proficiency in those techniques. Alternately, I was able to assist the lab staff with difficult or tedious analyses, or to observe analyses beyond my experience, such as atomic absorption spectrophotometry. Laboratory personnel were always willing to answer my questions and provide assistance in the lab.

PROCESS & TECHNIQUES LEARNED

Here I will list the analyses, processes and chemical techniques that I learned to perform during the internship. They are grouped under three headings: potable water tests (that is, fresh water suitable for human use and consumption), non-potable water tests (that is, water that the EPA has determined safe for release into the environment, such as industrial effluents and domestic wastewaters), and finally, miscellaneous tests (that is, tests related to marine science, water, or analytical chemistry in general).

The complete methods for all analyses listed, mostly taken from the United States E.P.A. (Environmental Protection Agency) publication "Methods for Chemical Analysis of Water & Wastes"¹, are given in the appendix, unless otherwise noted. All analyses (except S.C.U.B.A. air and soil pH) were performed in accordance with guidelines set by the E.P.A., including analyses taken from sources other than E.P.A. publications. In some cases, I have listed processes which are only part of an analysis; in such cases, the complete analysis method has been included in the appendix. The listing follows:

Potable Water Tests

- (i) Silica (dissolved, colorimetric) analysis
- (ii) Nitrogen, Nitrate-Nitrite analysis
- (iii) Suspended solids (non-filterable residue) analysis

- (iv) Biochemical oxygen demand (B.O.D.) analysis.
- (v) pH analysis of water samples.
- (vi) Preparation of water samples for iron analysis.
- (vii) Total dissolved solids (filterable residue) analysis.
- (viii) Hardness analysis (method from "Water and Wastewater Analysis Procedures," Hach Chemical Company²).
- (ix) Alkalinity analysis.
- (x) Total coliform analysis and bacterial media preparation (method from "Microbiological Methods for Monitoring the Environment," E.P.A. publication³).
- (xi) Fecal streptococci analysis (method from Microbiological Methods for Monitoring the Environment," E.P.A. publication³).
- (xii) Turbidity analysis.
- (xiii) Chloride analysis (method from "Water and Wastewater Analysis Procedures," Hach Chemical Company²).
- (xiv) Free chlorine colorimetric analysis is performed with the Hach portable field kit CN-46-A, which includes the method.

Non-Potable Water Tests

- (i) Suspended solids (filterable residue) analysis.
- (ii) Biochemical oxygen demand (B.O.D.) analysis.
- (iii) pH analysis of water samples.
- (iv) Total coliform analysis (method from "Microbiological Methods for Monitoring the Environment," E.P.A. publication³).
- (v) Fecal streptococci analysis (method from "Microbiological Methods for Monitoring the Environment," E.P.A. publication³).
- (vi) Oil and grease analysis.
- (vii) Settleable solids (residue, settleable matter) analysis.

Miscellaneous Tests

- (i) pH analysis of soil samples (method is not provided in appendix; it can be found in chapter 7 of "Soil Testing and Plant Analysis," Edited by L.M. Walsh and J.D. Beaton⁴)
- (ii) S.C.U.B.A. air analysis for carbon dioxide, carbon monoxide, and; oxygen (note: this analysis is performed in accordance with guidelines set by the State Department of Occupational Safety and Health; it is performed with Bendix colorimetric gas analysis tubes, distributed by N.E.I. Inc., P.O. Box 590 Warwick, R.I. 02888. The method is included with this apparatus, and is not provided in the appendix).
- (iii) Acid-base titrations for hydrogen peroxide analysis.
- (iv) Digestion of organic matter for arsenic analysis (method can be found in Perkin-Elmer atomic absorption spectrophotometer manual⁵, and is not provided in the appendix).
- (v) Digestion and preparation of taro and watercress (organic matter) for lead analysis (method can be found in Perkin-Elmer atomic absorption spectrophotometer manual⁵, and is not provided in the appendix).

The method for the preceding analyses appear in the appendix in the same order as they are listed here.

ADDITIONAL SKILLS GAINED

As well as specific analytical techniques in the area of water quality chemistry, I was exposed to many other useful aspects of commercial chemistry, inside and outside the laboratory. These include basic laboratory techniques common to many areas of laboratory science, such as dilutions and volumetric measurement, titration, filtration, drying, weighing, preparation of filter papers, sterile microbiological techniques, preparation of composite samples

for analysis, labware cleaning, and recovery/disposal of dangerous chemicals (for example, spilled mercury).

I was also able to observe laboratory staff perform analyses which are beyond my experience (such as atomic absorption spectrophotometry, P.A.B.A. analysis, cyanides analysis, and the analysis of a barge interior for oxygen deficiency and combustible gases by the marine chemist, whom I accompanied on the analysis) thereby gaining an understanding of these more difficult techniques which should prove useful in future work.

The Brewer Chemical Corporation places heavy emphasis on the safety of its employees and I feel that my awareness of the need for certain safety measures and my knowledge of laboratory safety procedures greatly increased during the internship. These safety procedures included fire-fighting (I attended a demonstration of fire-fighting procedure), proper laboratory attire and safe laboratory techniques in general.

Finally, after exposure to the various aforementioned drinking water and wastewater analyses(which, by law, must be satisfied by all potable and non-potable water) and the reading of E.P.A. publications ("Groundwater Protection"⁶ and "Primer for Wastewater Treatment"⁷) supplied by my advisor, I was able to appreciate how the E.P.A. attempts to (a) ensure that drinking water is safe, and (b) protect some of the United States' most valuable resources -- its inland waters, groundwater and the ocean -- from various pollutants, specifically: oxygen demanding wastes, disease-causing agents, plant nutrients, synthetic organic chemicals, inorganic chemicals and mineral substances, and sediments.

ACKNOWLEDGEMENTS

First, I would like to thank the Brewer Chemical Corporation for providing the internship. I would also like to acknowledge the excellent training, constant assistance, patience and moral support of Estelle Shiroma

(Laboratory Supervisor, Brewer Analytical Laboratories -- my advisor), Erwin Kawata, Greg Uyechi and Mike Schmidt (Brewer Analytical Laboratory staff) and Ken Chang (Marine Chemist). Thanks also to Barb Lee, Manoa M.O.P. Coordinator, for help with this report.

I was provided with a student stipend of one hundred dollars per month during the internship as well as travelling costs by the UH-Manoa Marine Option Program from its Sea Grant Funds.

Evaluation

Overall, I found this internship to be an interesting, enjoyable and extremely worthwhile experience. My objectives were satisfied in that I gained some practical experience in my intended field of study outside of the University situation, furthered my knowledge of marine-related analytical chemistry and acquired additional skills and knowledge in the area of professional chemistry.

REFERENCES

1. "Methods for Chemical Analysis of Water and Wastes," Environmental Monitoring and Support Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, Ohio; March 1979 (460 pages).
2. "Water and Wastewater Analysis Procedures," Hach Chemical Company, Ames, Iowa; August 1967 (104 pages).
3. "Microbiological Methods for Monitoring the Environment - Water and Wastes," Environment Monitoring and Support Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, Ohio; December 1978 (354 pages).
4. "Soil Testing and Plant Analysis," Edited by L.M. Walsh and J.D. Beaton, Soil Science Society of America, Inc., Madison, Wisconsin; 1973 (491 pages).
5. "Analytical Methods for Atomic Absorption Spectrophotometry," Perkin-Elmer Corporation, Norwalk, Connecticut; September 1976 (300 pages).
6. "Groundwater Protection," Edited by J.A. Davis, Water Planning Division and Office of Solid Waste, U.S. Environmental Protection Agency, Washington, D.C.; November 1980 (36 pages).
7. "Primer for Wastewater Treatment," Office of Water, Programs Operations, U.S. Environmental Protection Agency, Washington D.C.; July 1980 (20 pages).

APPENDIX A: Potable Water Tests

- (i) Silica (dissolved, colorimetric)
- (ii) Nitrogen, nitrate-nitrite
- (iii) Suspended solids (non-filterable residue)
- (iv) Biochemical oxygen demand (B.O.D.)
- (v) pH analysis of water samples
- (vi) Preparation of water samples for iron analysis
- (vii) Total dissolved solids (filterable residue)
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- (ix) Alkalinity analysis
- (x) Total coliform analysis and bacterial media preparation
(method from "Microbiological Methods for Monitoring the Environment," E.P.A. publication)
- (xi) Fecal streptococci analysis (method from Microbiological Methods for Monitoring the Environment," E.P.A. publication)
- (xii) Turbidity analysis
- (xiii) Chloride analysis (method from "Water and Wastewater Analysis Procedures," Hach Chemical Company)

SILICA, DISSOLVED

Method 370.1 (Colorimetric)

STORET NO. Dissolved 00955

1. Scope and Application
 - 1.1 This method is applicable to drinking, surface and saline waters, domestic and industrial wastes.
 - 1.2 The working range of the method is approximately 2 to 25 mg silica/l. The upper range can be extended by taking suitable aliquots; the lower range can be extended by the addition of amino-naphthol-sulfonic acid solution, as described in (6.8).
2. Summary of Method
 - 2.1 A well-mixed sample is filtered through a 0.45 μ membrane filter. The filtrate, upon the addition of molybdate ion in acidic solution, forms a greenish-yellow color complex proportional to the dissolved silica in the sample. The color complex is then measured spectrophotometrically.
 - 2.2 In the low concentration modification the yellow (410 nm) molybdosilicic acid color is reduced by 1-amino-2-naphthol-4-sulfonic acid to a more intense heteropoly blue (815 nm or 650 nm).
3. Interferences
 - 3.1 Excessive color and/or turbidity interfere. Correct by running blanks prepared without addition of the ammonium molybdate solution. See (6.7).
 - 3.2 Tannin interference may be eliminated and phosphate interferences may be decreased with oxalic acid.
 - 3.3 Large amounts of iron and sulfide interfere.
 - 3.4 Contact with glass should be minimized, silica free reagents should be used as much as possible. A blank should be run.
4. Apparatus
 - 4.1 Platinum dishes, 100 ml.
 - 4.2 Colorimetric equipment—one of the following:
 - 4.2.1 Spectrophotometer for use at 410 nm, 650 nm and/or 815 nm with a 1 cm or longer cell.
 - 4.2.2 Filter photometer with a violet filter having maximum transmittance as near 410 nm as possible and a 1 cm or longer cell.
 - 4.2.3 Nessler tubes, matched, 50 ml, tall form.
5. Reagents
 - 5.1 Use chemicals low in silica and store in plastic containers.
 - 5.2 Sodium bicarbonate, NaHCO_3 , powder.
 - 5.3 Sulfuric acid, H_2SO_4 , 1 N.

Approved for NPDES

Issued 1971

Editorial revision 1978

- 5.4 Hydrochloric acid, HCl, 1 + 1.
- 5.5 Ammonium molybdate reagent: Place 10 g $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$ in distilled water in a 100 ml volumetric. Dissolve by stirring and gently warming. Dilute to the mark. Filter if necessary. Adjust to pH 7 to 8 with silica free NH_4OH or NaOH . Store in plastic bottle.
- 5.6 Oxalic acid solution: Dissolve 10 g $\text{H}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$ in distilled water in a 100 ml volumetric flask, dilute to the mark. Store in plastic.
- 5.7 Stock silica solution: Dissolve 4.73 g sodium metasilicate nonahydrate, $\text{Na}_2\text{SiO}_3 \cdot 9\text{H}_2\text{O}$, in recently boiled and cooled distilled water. Dilute to approximately 900 ml. Analyze 100.0 ml portions by gravimetry (ref. 1, p. 484). Adjust concentration to 1.000 mg/1 SiO_2 . Store in tightly stoppered plastic bottle.
- 5.8 Standard silica solution: Dilute 10.0 ml stock solution to 1 liter with recently boiled and cooled distilled water. This is 10 mg/1 SiO_2 (1.00 ml = 10.0 μg SiO_2). Store in a tightly stoppered plastic bottle.
- 5.9 Permanent color solutions
 - 5.9.1 Potassium chromate solution: Dissolve 630 mg K_2CrO_4 in distilled water in a 1 liter volumetric flask and dilute to the mark.
 - 5.9.2 Borax solution: Dissolve 10 g sodium borate decahydrate, $(\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O})$ in distilled water in a 1 liter volumetric flask and dilute to the mark.
- 5.10 Reducing agent: Dissolve 500 mg of 1-amino-2-naphthol-4-sulfonic acid and 1 g Na_2SO_3 in 50 ml distilled water with gentle warming if necessary. Dissolve 30 g NaHSO_3 in 150 ml distilled water. Mix these two solutions. Filter into a plastic bottle. Refrigerate and avoid exposure to light. Discard when it darkens. If there is incomplete solubility or immediate darkening of the aminonaphthosulfonic acid solution do not use.

6. Procedure

- 6.1 Filter sample through a 0.45 μ membrane filter.
- 6.2 Digestion: If molybdate unreactive silica is present and its inclusion in the analysis is desired, include this step, otherwise proceed to 6.3.
 - 6.2.1 Place 50 ml, or a smaller portion diluted to 50 ml, of filtered (6.1) sample in a 100 ml platinum dish.
 - 6.2.2 Add 200 mg silica-free NaHCO_3 (5.2) and digest on a steam bath for 1 hour. Cool.
 - 6.2.3 Add slowly and with stirring 2.4 ml H_2SO_4 (5.3).
 - 6.2.4 Immediately transfer to a 50 ml Nessler tube, dilute to the mark with distilled water and proceed to 6.3 without delay.
- 6.3 Color development
 - 6.3.1 Place 50 ml sample in a Nessler tube.
 - 6.3.2 Add rapidly 1.0 ml of 1 + 1 HCl (5.4) and 2.0 ml ammonium molybdate reagent (5.5).
 - 6.3.3 Mix by inverting at least 6 times.
 - 6.3.4 Let stand 5 to 10 minutes.
 - 6.3.5 Add 1.5 ml oxalic acid solution (5.6) and mix thoroughly.
 - 6.3.6 Read color (spectrophotometrically or visually) after 2 minutes but before 15 minutes from the addition of oxalic acid.

6.4 Preparation of Standards

6.4.1 If digestion (6.2) was used add 200 mg NaHCO_3 (5.2) and 2.4 ml H_2SO_4 (5.3) to standards to compensate for silica introduced by these reagents and for effect of the salt on the color intensity.

6.5 Photometric measurement

6.5.1 Prepare a calibration curve using approximately six standards to span the range shown below with the selected light path.

Selection of Light Path Length for Various
Silica Concentrations

Light Path cm	Silica in 54.5 ml final volume (μg)
1	200-1300
2	100-700
5	40-250
10	20-130

6.5.2 Carry out the steps in 6.3 using distilled water as the reference. Read a blank.

6.5.3 Plot photometric reading versus μg of silica in the final solution of 54.5 ml. Run a reagent blank and at least one standard with each group of samples.

6.6 Visual Comparison

6.6.1 Prepare a set of permanent artificial color standards according to the table. Use well stoppered, properly labelled 50 ml Nessler tubes.

Silica value mg	Potassium chromate solution (5.9.1) ml	Borax solution (5.9.2) ml	Distilled water ml
0.00	0.0	25	30
0.10	1.0	25	29
0.20	2.0	25	28
0.40	4.0	25	26
0.50	5.0	25	25
0.75	7.5	25	22
1.00	10.0	25	20

6.6.2 Verify permanent standards by comparison to color developed by standard silica solutions.

6.6.3 These permanent artificial color standards are only for color comparison procedure, not for photometric procedure.

6.7 Correction for color or turbidity

6.7.1 A special blank is run using a portion of the sample and carrying out the procedure in 6.1, 6.2 if used, and 6.3 except for the addition of ammonium molybdate (6.3.2).

- 6.7.2 Zero the photometer with this blank before reading the samples.
- 6.8 Procedure for low concentration ($< 1000 \text{ ug/l}$)
- 6.8.1 Perform steps 6.1 and 6.2 if needed.
- 6.8.2 Place 50 ml sample in a Nessler tube.
- 6.8.3 In rapid succession add 1.0 ml of 1 + 1 HCl (5.4).
- 6.8.4 Add 2.0 ml ammonium molybdate reagent (5.5).
- 6.8.5 Mix by inverting at least six times.
- 6.8.6 Let stand 5 to 10 minutes.
- 6.8.7 Add 1.5 ml oxalic acid solution (5.6).
- 6.8.8 Mix thoroughly.
- 6.8.9 At least 2, but not more than 15 minutes after oxalic acid addition, add 2.0 ml reducing agent (5.10).
- 6.8.10 Mix thoroughly.
- 6.8.11 Wait 5 minutes, read photometrically or visually.
- 6.8.12 If digestion (6.2) was used see (6.4).
- 6.8.13 Photometric measurement

- 6.8.13.1 Prepare a calibration curve using approximately 6 standards and a reagent blank to span the range shown below with the selected light path.

Selection of Light Path Length for
Various Silica Concentrations

Light Path cm	Silica in 56.5 ml Final volume, ug	
	650 nm	815 nm
1	40-300	20-100
2	20-150	10-50
5	7-50	4-20
10	4-30	2-10

- 6.8.13.2 Read versus distilled water.
- 6.8.13.3 Plot photometric reading at 650 nm or at 815 nm versus ug of silica in 56.5 ml.
- 6.8.13.4 For turbidity correction use 6.1, 6.2 if used and 6.8.2-6.8.11 omitting 6.8.4 and 6.8.9.
- 6.8.13.5 Run a reagent blank and at least one standard (to check calibration curve drift) with each group of samples.
- 6.8.14 Visual comparison
- 6.8.14.1 Prepare not less than 12 standards covering the range of 0 to 120 ug SiO_2 by placing the calculated volumes of standard silica (5.8) in 50 ml Nessler tubes, diluting to the mark and develop the color as in 6.8.2-6.8.11.

7. Calculations

7.1 Read $\mu\text{g SiO}_2$ from calibration curve or by visual comparison

$$7.2 \text{ mg/l SiO}_2 = \frac{\mu\text{g/SiO}_2}{\text{ml sample}}$$

7.3 Report whether NaHCO_3 digestion (6.2) was used

8. Precision and Accuracy

8.1 A synthetic unknown sample containing 5.0 mg/l SiO_2 , 10 mg/l chloride, 0.200 mg/l ammonia N, 1.0 mg/l nitrate N, 1.5 mg/l organic N, and 10.0 mg/l phosphate in distilled water was analyzed in 19 laboratories by the molybdosilicate method, with a relative standard deviation of 14.3% and a relative error of 7.8%.

8.2 Another synthetic unknown sample containing 15.0 mg/l SiO_2 , 200 mg/l chloride, 0.800 mg/l ammonia N, 1.0 mg/l nitrate N, 0.800 mg/l organic N, and 5.0 mg/l phosphate in distilled water was analyzed in 19 laboratories by the molybdosilicate method, with a relative standard deviation of 8.4% and a relative error of 4.2%.

8.3 A third synthetic unknown sample containing 30.0 mg/l SiO_2 , 400 mg/l chloride, 1.50 mg/l ammonia N, 1.0 mg/l nitrate N, 0.200 mg/l organic N, and 0.500 mg/l phosphate in distilled water was analyzed in 20 laboratories by the molybdosilicate method, with a relative standard deviation of 7.7% and a relative error of 9.8%. All results were obtained after sample digestion with NaHCO_3 .

8.4 Photometric evaluations by the amino-naphthol-sulfonic acid procedure have an estimated precision of ± 0.10 mg/l in the range from 0 to 2 mg/l (ASTM).

8.5 Photometric evaluations of the silico-molybdate color in the range from 2 to 50 mg/l have an estimated precision of approximately 4% of the quantity of silica measured (ASTM).

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1. Annual Book of ASTM Standards, Part 31, "Water", Standard D859-68, p 401 (1976).
2. Standard Methods for the Examination of Water and Wastewater, 14th Edition, p 487, Method 426B, (1975).

NITROGEN, NITRATE-NITRITE

Method 353.3 (Spectrophotometric, Cadmium Reduction)

STORET NO. Total 00630

1. Scope and Application
 - 1.1 This method is applicable to the determination of nitrite singly, or nitrite and nitrate combined in drinking, surface and saline waters, domestic and industrial wastes. The applicable range of this method is 0.01 to 1.0 mg/1 nitrate-nitrite nitrogen. The range may be extended with sample dilution.
2. Summary of Method
 - 2.1 A filtered sample is passed through a column containing granulated copper-cadmium to reduce nitrate to nitrite. The nitrite (that originally present plus reduced nitrate) is determined by diazotizing with sulfanilamide and coupling with N-(1-naphthyl)-ethylenediamine dihydrochloride to form a highly colored azo dye which is measured spectrophotometrically. Separate, rather than combined nitrate-nitrite, values are readily obtained by carrying out the procedure first with, and then without, the Cu-Cd reduction step.
3. Sample Handling and Preservation
 - 3.1 Analysis should be made as soon as possible. If analysis can be made within 24 hours, the sample should be preserved by refrigeration at 4°C. When samples must be stored for more than 24 hours, they should be preserved with sulfuric acid (2 ml H₂SO₄ per liter) and refrigeration.

Caution: Samples for reduction column must not be preserved with mercuric chloride.
4. Interferences
 - 4.1 Build up of suspended matter in the reduction column will restrict sample flow. Since nitrate-nitrogen is found in a soluble state, the sample may be pre-filtered through a glass fiber filter or a 0.45 μ membrane filter. Highly turbid samples may be pretreated with zinc sulfate before filtration to remove the bulk of particulate matter present in the sample.
 - 4.2 Low results might be obtained for samples that contain high concentrations of iron, copper or other metals. EDTA is added to the samples to eliminate this interference.
 - 4.3 Samples that contain large concentrations of oil and grease will coat the surface of the cadmium. This interference is eliminated by pre-extracting the sample with an organic solvent.
 - 4.4 This procedure determines both nitrate and nitrite. If only nitrate is desired, a separate determination must be made for nitrite and subsequent corrections made. The nitrite may be determined by the procedure below without the reduction step.

5. Apparatus

5.1 Reduction column: The column in Figure I was constructed from a 100 ml pipet by removing the top portion. This column may also be constructed from two pieces of tubing joined end to end. A 10 mm length of 3 cm I.D. tubing is joined to a 25 cm length of 3.5 mm I.D. tubing.

5.2 Spectrophotometer for use at 540 nm, providing a light path of 1 cm or longer.

6. Reagents

6.1 Granulated cadmium: 40–60 mesh (E M Laboratories, Inc., 500 Exec. Blvd., Elmsford, NY 10523, Cat. 2001 Cadmium, Coarse Powder).

6.2 Copper-Cadmium: The cadmium granules (new or used) are cleaned with dilute HCl and copperized with 2% solution of copper sulfate in the following manner:

6.2.1 Wash the cadmium with dilute HCl (6.10) and rinse with distilled water. The color of the cadmium should be silver.

6.2.2 Swirl 25 g cadmium in 100 ml portions of a 2% solution of copper sulfate (6.11) for 5 minutes or until blue color partially fades, decant and repeat with fresh copper sulfate until a brown colloidal precipitate forms.

6.2.3 Wash the copper-cadmium with distilled water (at least 10 times) to remove all the precipitated copper. The color of the cadmium so treated should be black.

6.3 Preparation of reaction column: Insert a glass wool plug into the bottom of the reduction column and fill with distilled water. Add sufficient copper-cadmium granules to produce a column 18.5 cm in length. Maintain a level of distilled water above the copper-cadmium granules to eliminate entrapment of air. Wash the column with 200 ml of dilute ammonium chloride solution (6.5). The column is then activated by passing through the column 100 ml of a solution composed of 25 ml of a 1.0 mg/1 $\text{NO}_3\text{-N}$ standard and 75 ml of ammonium chloride – EDTA solution (6.4). Use a flow rate between 7 and 10 ml per minute.

6.4 Ammonium chloride – EDTA solution: Dissolve 13 g ammonium chloride and 1.7 g disodium ethylenediamine tetracetate in 900 ml of distilled water. Adjust the pH to 8.5 with conc. ammonium hydroxide (6.9) and dilute to 1 liter.

6.5 Dilute ammonium chloride-EDTA solution: Dilute 300 ml of ammonium chloride-EDTA solution (6.4) to 500 ml with distilled water.

6.6 Color reagent: Dissolve 10 g sulfanilamide and 1 g N(1-naphthyl)-ethylene-diamine dihydrochloride in a mixture of 100 ml conc. phosphoric acid and 800 ml of distilled water and dilute to 1 liter with distilled water.

6.7 Zinc sulfate solution: Dissolve 100 g $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in distilled water and dilute to 1 liter.

6.8 Sodium hydroxide solution, 6N: Dissolve 240 g NaOH in 500 ml distilled water, cool and dilute to 1 liter.

6.9 Ammonium hydroxide, conc.

6.10 Dilute hydrochloric acid, 6N: Dilute 50 ml of conc. HCl to 100 ml with distilled water.

6.11 Copper sulfate solution, 2%: Dissolve 20 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 500 ml of distilled water and dilute to 1 liter.

6.12 Stock nitrate solution: Dissolve 7.218 g KNO_3 in distilled water and dilute to 1000 ml. Preserve with 2 ml of chloroform per liter. This solution is stable for at least 6 months. 1.0 ml = 1.00 mg $\text{NO}_3\text{-N}$.

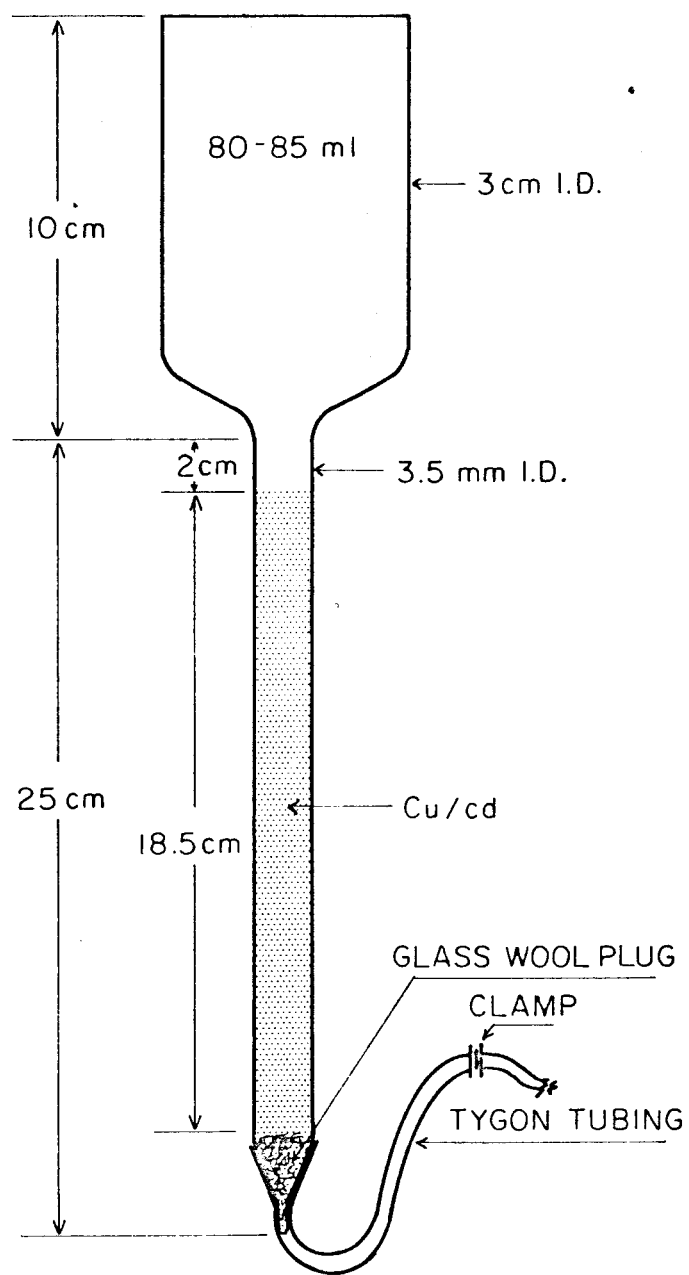


FIGURE 1. REDUCTION COLUMN

- 6.13 Standard nitrate solution: Dilute 10.0 ml of nitrate stock solution (6.12) to 1000 ml with distilled water. 1.0 ml = 0.01 mg $\text{NO}_3\text{-N}$.
- 6.14 Stock nitrite solution: Dissolve 6.072 g KNO_2 in 500 ml of distilled water and dilute to 1000 ml. Preserve with 2 ml of chloroform and keep under refrigeration. Stable for approximately 3 months. 1.0 ml = 1.00 mg $\text{NO}_2\text{-N}$.
- 6.15 Standard nitrite solution: Dilute 10.0 ml of stock nitrite solution (6.14) to 1000 ml with distilled water. 1.0 ml = 0.01 mg $\text{NO}_2\text{-N}$.
- 6.16 Using standard nitrate solution (6.13) prepare the following standards in 100 ml volumetric flasks:

<u>Conc., mg-$\text{NO}_3\text{-N/l}$</u>	<u>ml of Standard Solution/100.0 ml</u>
0.00	0.0
0.05	0.5
0.10	1.0
0.20	2.0
0.50	5.0
1.00	10.0

7. Procedure

- 7.1 Turbidity removal: One of the following methods may be used to remove suspended matter.
- 7.1.1 Filter sample through a glass fiber filter or a 0.45 μ membrane filter.
- 7.1.2 Add 1 ml zinc sulfate solution (6.7) to 100 ml of sample and mix thoroughly. Add 0.4–0.5 ml sodium hydroxide solution (6.8) to obtain a pH of 10.5 as determined with a pH meter. Let the treated sample stand a few minutes to allow the heavy flocculent precipitate to settle. Clarify by filtering through a glass fiber filter or a 0.45 μ membrane filter.
- 7.2 Oil and grease removal: Adjust the pH of 100 ml of filtered sample to 2 by addition of conc. HCl. Extract the oil and grease from the aqueous solution with two 25 ml portions of a non-polar solvent (Freon, chloroform or equivalent).
- 7.3 If the pH of the sample is below 5 or above 9, adjust to between 5 and 9 with either conc. HCl or conc. NH_4OH . This is done to insure a sample pH of 8.5 after step 7.4.
- 7.4 To 25.0 ml of sample or an aliquot diluted to 25.0 ml, add 75 ml of ammonium chloride-EDTA solution (6.4) and mix.
- 7.5 Pour sample into column and collect sample at a rate of 7–10 ml per minute.
- 7.6 Discard the first 25 ml, collect the rest of the sample (approximately 70 ml) in the original sample flask. Reduced samples should not be allowed to stand longer than 15 minutes before addition of color reagent, step 7.7.
- 7.7 Add 2.0 ml of color reagent (6.6) to 50.0 ml of sample. Allow 10 minutes for color development. Within 2 hours measure the absorbance at 540 nm against a reagent blank.
- NOTE:** If the concentration of sample exceeds 1.0 mg $\text{NO}_3\text{-N/l}$, the remainder of the reduced sample may be used to make an appropriate dilution before proceeding with step 7.7.

7.8 Standards: Carry out the reduction of standards exactly as described for the samples. At least one nitrite standard should be compared to a reduced nitrate standard at the same concentration to verify the efficiency of the reduction column.

8. Calculation

8.1 Obtain a standard curve by plotting the absorbance of standards run by the above procedure against $\text{NO}_3\text{-N}$ mg/l. Compute concentration of samples by comparing sample absorbance with standard curve.

8.2 If less than 25 ml of sample is used for the analysis the following equation should be used:

$$\text{mgNO}_2 + \text{NO}_3 - \text{N/l} = \frac{A \times 25}{\text{ml sample used}}$$

where:

A = Concentration of nitrate from standard curve.

9. Precision and Accuracy

9.1 In a single laboratory (EMSL), using sewage samples at concentrations of 0.04, 0.24, 0.55 and 1.04 mg $\text{NO}_3 + \text{NO}_2\text{-N/l}$, the standard deviations were ± 0.005 , ± 0.004 , ± 0.005 and ± 0.01 , respectively.

9.2 In a single laboratory (EMSL), using sewage samples at concentrations of 0.24, 0.55, and 1.05 mg $\text{NO}_3 + \text{NO}_2\text{-N/l}$, the recoveries were 100%, 102% and 100%, respectively.

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(SUSPENDED)

RESIDUE, NON-FILTERABLE

Method 160.2 (Gravimetric, Dried at 103–105°C)

STORET NO. 00530

1. Scope and Application
 - 1.1 This method is applicable to drinking, surface, and saline waters, domestic and industrial wastes.
 - 1.2 The practical range of the determination is 4 mg/l to 20,000 mg/l.
2. Summary of Method
 - 2.1 A well-mixed sample is filtered through a glass fiber filter, and the residue retained on the filter is dried to constant weight at 103–105°C.
 - 2.2 The filtrate from this method may be used for Residue, Filterable.
3. Definitions
 - 3.1 Residue, non-filterable, is defined as those solids which are retained by a glass fiber filter and dried to constant weight at 103–105°C.
4. Sample Handling and Preservation
 - 4.1 Non-representative particulates such as leaves, sticks, fish, and lumps of fecal matter should be excluded from the sample if it is determined that their inclusion is not desired in the final result.
 - 4.2 Preservation of the sample is not practical; analysis should begin as soon as possible. Refrigeration or icing to 4°C, to minimize microbiological decomposition of solids, is recommended.
5. Interferences
 - 5.1 Filtration apparatus, filter material, pre-washing, post-washing, and drying temperature are specified because these variables have been shown to affect the results.
 - 5.2 Samples high in Filterable Residue (dissolved solids), such as saline waters, brines and some wastes, may be subject to a positive interference. Care must be taken in selecting the filtering apparatus so that washing of the filter and any dissolved solids in the filter (7.5) minimizes this potential interference.
6. Apparatus
 - 6.1 Glass fiber filter discs, without organic binder, such as Millipore AP-40, Reeves Angel 934-AH, Gelman type A/E, or equivalent.

NOTE: Because of the physical nature of glass fiber filters, the absolute pore size cannot be controlled or measured. Terms such as "pore size", collection efficiencies and effective retention are used to define this property in glass fiber filters. Values for these parameters vary for the filters listed above.
 - 6.2 Filter support: filtering apparatus with reservoir and a coarse (40–60 microns) fritted disc as a filter support.

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Issued 1971

NOTE: Many funnel designs are available in glass or porcelain. Some of the most common are Hirsch or Buchner funnels, membrane filter holders and Gooch crucibles. All are available with coarse fritted disc.

- 6.3 Suction flask.
- 6.4 Drying oven, 103–105°C.
- 6.5 Desiccator.
- 6.6 Analytical balance, capable of weighing to 0.1 mg.

7. Procedure

- 7.1 Preparation of glass fiber filter disc: Place the glass fiber filter on the membrane filter apparatus or insert into bottom of a suitable Gooch crucible with wrinkled surface up. While vacuum is applied, wash the disc with three successive 20 ml volumes of distilled water. Remove all traces of water by continuing to apply vacuum after water has passed through. Remove filter from membrane filter apparatus or both crucible and filter if Gooch crucible is used, and dry in an oven at 103–105°C for one hour. Remove to desiccator and store until needed. Repeat the drying cycle until a constant weight is obtained (weight loss is less than 0.5 mg). Weigh immediately before use. After weighing, handle the filter or crucible/filter with forceps or tongs only.

7.2 Selection of Sample Volume

For a 4.7 cm diameter filter, filter 100 ml of sample. If weight of captured residue is less than 1.0 mg, the sample volume must be increased to provide at least 1.0 mg of residue. If other filter diameters are used, start with a sample volume equal to 7 ml/cm² of filter area and collect at least a weight of residue proportional to the 1.0 mg stated above.

NOTE: If during filtration of this initial volume the filtration rate drops rapidly, or if filtration time exceeds 5 to 10 minutes, the following scheme is recommended: Use an unweighed glass fiber filter of choice affixed in the filter assembly. Add a known volume of sample to the filter funnel and record the time elapsed after selected volumes have passed through the filter. Twenty-five ml increments for timing are suggested. Continue to record the time and volume increments until filtration rate drops rapidly. Add additional sample if the filter funnel volume is inadequate to reach a reduced rate. Plot the observed time versus volume filtered. Select the proper filtration volume as that just short of the time a significant change in filtration rate occurred.

- 7.3 Assemble the filtering apparatus and begin suction. Wet the filter with a small volume of distilled water to seat it against the fritted support.
- 7.4 Shake the sample vigorously and quantitatively transfer the predetermined sample volume selected in 7.2 to the filter using a graduated cylinder. Remove all traces of water by continuing to apply vacuum after sample has passed through.
- 7.5 With suction on, wash the graduated cylinder, filter, non-filterable residue and filter funnel wall with three portions of distilled water allowing complete drainage between washing. Remove all traces of water by continuing to apply vacuum after water has passed through.

NOTE: Total volume of wash water used should equal approximately 2 ml per cm². For a 4.7 cm filter the total volume is 30 ml.

7.6. Carefully remove the filter from the filter support. Alternatively, remove crucible and filter from crucible adapter. Dry at least one hour at 103–105°C. Cool in a desiccator and weigh. Repeat the drying cycle until a constant weight is obtained (weight loss is less than 0.5 mg).

8. Calculations

8.1. Calculate non-filterable residue as follows:

$$\text{Non-filterable residue, mg/l} = \frac{(A - B) \times 1,000}{C}$$

where:

A = weight of filter (or filter and crucible) + residue in mg

B = weight of filter (or filter and crucible) in mg

C = ml of sample filtered

9. Precision and Accuracy

9.1 Precision data are not available at this time.

9.2 Accuracy data on actual samples cannot be obtained.

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BIOCHEMICAL OXYGEN DEMAND

Method 405.1 (5 Days, 20°C)

STORET NO. 00310

1. Scope and Application
 - 1.1 The biochemical oxygen demand (BOD) test is used for determining the relative oxygen requirements of municipal and industrial wastewaters. Application of the test to organic waste discharges allows calculation of the effect of the discharges on the oxygen resources of the receiving water. Data from BOD tests are used for the development of engineering criteria for the design of wastewater treatment plants.
 - 1.2 The BOD test is an empirical bioassay-type procedure which measures the dissolved oxygen consumed by microbial life while assimilating and oxidizing the organic matter present. The standard test conditions include dark incubation at 20°C for a specified time period (often 5 days). The actual environmental conditions of temperature, biological population, water movement, sunlight, and oxygen concentration cannot be accurately reproduced in the laboratory. Results obtained must take into account the above factors when relating BOD results to stream oxygen demands.
2. Summary of Method
 - 2.1 The sample of waste, or an appropriate dilution, is incubated for 5 days at 20°C in the dark. The reduction in dissolved oxygen concentration during the incubation period yields a measure of the biochemical oxygen demand.
3. Comments
 - 3.1 Determination of dissolved oxygen in the BOD test may be made by use of either the Modified Winkler with Full-Bottle Technique or the Probe Method in this manual.
 - 3.2 Additional information relating to oxygen demanding characteristics of wastewaters can be gained by applying the Total Organic Carbon and Chemical Oxygen Demand tests (also found in this manual).
 - 3.3 The use of 60 ml incubation bottles in place of the usual 300 ml incubation bottles, in conjunction with the probe, is often convenient.
4. Precision and Accuracy
 - 4.1 Eighty-six analysts in fifty-eight laboratories analyzed natural water samples plus an exact increment of biodegradable organic compounds. At a mean value of 2.1 and 175 mg/l BOD, the standard deviation was ± 0.7 and ± 26 mg/l, respectively (EPA Method Research Study 3).
 - 4.2 There is no acceptable procedure for determining the accuracy of the BOD test.

Approved for NPDES
Issued 1971
Editorial revision 1974

5. References

- 5.1 The procedure to be used for this determination is found in:
Standard Methods for the Examination of Water and Wastewater, 14th Edition, p 543,
Method 507 (1975).

1. Discussion

The biochemical oxygen demand (BOD) determination described herein is an empirical test in which standardized laboratory procedures are used to determine the relative oxygen requirements of wastewaters, effluents, and polluted waters. The test has its widest application in measuring waste loadings to treatment plants and in evaluating the efficiency (BOD removal) of such treat-

ment systems. BOD values cannot be compared unless the results have been obtained under identical test conditions.

The test is of limited value in measuring the actual oxygen demand of surface waters. The extrapolation of test results to actual stream oxygen demands is highly questionable because the laboratory environment does not reproduce stream conditions such as temperature, sunlight, biological population, water movement, and oxygen concentration.

Samples for BOD analysis may undergo significant degradation during

*BOD, biochemical oxygen demand.

handling and storage. Some of the demand may be satisfied if the sample is held for several days before the test is initiated; this results in a low estimation of the true BOD. The extent of change appears to be a function of the amount of organic matter (food supply) and the number and types of organisms (biological population). To reduce the change in oxygen demand that occurs between sampling and testing, keep all samples at or below 4 C and begin incubation not more than 24 hr after the sample is collected.

The amount of oxygen demand in the sample will govern the need for and the degree of dilution.

Aerate samples with low DO values to increase the initial DO content above that required by the BOD. Let air bubble through a diffusion tube into the sample for 5 min, or until the DO is at least 7 mg/l. Determine DO on one portion of the aerated sample; seed another portion only if necessary, and incubate it for the BOD determination.

Complete stabilization of a given waste may require a period of incubation too long for practical purposes. For this reason, the 5-day period has been accepted as standard. However, for certain industrial wastes it may be advisable to determine the oxidation curve obtained. Conversion of data from one incubation period to another can be made only if such special studies are carried out. Studies in recent years have shown that the exponential rate of carbonaceous oxidation, k , at 20 C rarely has a value of 0.1, although it may vary from less than one-half to more than twice this value. This fact usually makes it impossible to calculate the ultimate carbonaceous demand, L , of a sample from 5-day BOD values unless the k

value has been determined on the sample. The exponential interpretation of BOD rate curves is a gross oversimplification; a good exponential fit is not obtained always.

The test measures the oxygen demand produced by carbonaceous and nitrogenous compounds, and immediate oxidation. All of these have a bearing on the oxygen balance of the receiving water and must be considered in the discharge of a waste to such water. Differentiation of the immediate dissolved oxygen demand is described in ¶4j below. Appropriate technics for the suppression of nitrification in tests for carbonaceous demand only are given elsewhere.¹⁻⁵ If nitrification suppression is used, state this clearly when reporting results. Bear in mind that some suppressors may also inhibit carbonaceous oxidation.

2. Apparatus

a. Incubation bottles, 250- to 300-ml capacity, with ground-glass stoppers. Clean bottles with a good detergent, rinse thoroughly, and drain before use. As a precaution against drawing air into the dilution bottle during incubation, use a water seal. Satisfactory water seals are obtained by inverting the bottles in a water bath or adding water to the flared mouth of special BOD bottles.

b. Air incubator or water bath, thermostatically controlled at $20\text{ C} \pm 1\text{ C}$. Exclude all light to prevent formation of DO by algae in the sample.

3. Reagents

a. Distilled water: Use only high-quality water distilled from a block tin or all-glass still. Alternatively, use de-

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ionized water. The water must contain less than 0.01 mg/l copper, and be free of chlorine, chloramines, caustic alkalinity, organic material, or acids.

b. Phosphate buffer solution: Dissolve 8.5 g potassium dihydrogen phosphate, KH_2PO_4 ; 21.75 g dipotassium hydrogen phosphate, K_2HPO_4 ; 33.4 g disodium hydrogen phosphate heptahydrate, $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$; and 1.7 g ammonium chloride, NH_4Cl , in about 500 ml distilled water and dilute to 1 l. The pH of this buffer should be 7.2 without further adjustment. Discard the reagent (or any of the following reagents) if there is any sign of biological growth in the stock bottle.

c. Magnesium sulfate solution: Dissolve 22.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in distilled water and dilute to 1 l.

d. Calcium chloride solution: Dissolve 27.5 g anhydrous CaCl_2 in distilled water and dilute to 1 l.

e. Ferric chloride solution: Dissolve 0.25 g $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in distilled water and dilute to 1 l.

f. Acid and alkali solutions, 1N: For neutralization of caustic or acidic waste samples.

g. Sodium sulfite solution, 0.025N: Dissolve 1.575 g anhydrous Na_2SO_3 in 1,000 ml distilled water. This solution is not stable; prepare daily.

b. Seeding: The purpose of seeding is to introduce into the sample a biological population capable of oxidizing the organic matter in the wastewater. Where such microorganisms are already present, as in domestic wastewater or unchlorinated effluents and surface waters, seeding is unnecessary and should not be used.

When the sample contains very few microorganisms—as a result, for ex-

ample, of chlorination, high temperature, or extreme pH—seed the dilution water. The standard seed material is settled domestic wastewater that has been stored at 20 C for 24 to 36 hr. Use sufficient seed to produce a seed correction (¶4g) of at least 0.6 mg/l.

Some samples—for example, certain industrial wastes—may require seeding because of low microbial population, but they contain organic compounds that are not readily oxidized by domestic wastewater seed. For evaluating the effect of such a waste in a treatment system, it is better to use specialized seed material containing organisms adapted to the use of the organic compounds present. Obtain such adapted seed from the effluent of a biological treatment process receiving the waste in question, or from the receiving water below the point of discharge [preferably 3 to 8 km (2 to 5 miles) below] if the waste is not being treated. When these sources are not available, develop adapted seed in the laboratory by continuously aerating a large sample of water and feeding it with small daily increments of the particular waste, together with soil or domestic sewage, until a satisfactory microbial population has developed. The special circumstances that call for the use of adapted seed also may require a seed concentration higher than the standard 1 to 2 ml/l. Decide on the kind and amount of seed required for such special-purpose studies on the basis of prior experience with the particular waste and the purpose for which the determination is being made.

Adapted seed also has been used in attempts to estimate the effect of a waste on the receiving water. (See Section 507.1).

4. Procedure

a. Preparation of dilution water: Before use, store the distilled water in cotton-plugged bottles long enough for it to become saturated with DO; or, if such storage is not practical, saturate the water by shaking the partially filled bottle or by aerating with a supply of clean compressed air. Use distilled water at 20 ± 1 C.

Place the desired volume of distilled water in a suitable bottle and add 1 ml each of phosphate buffer, MgSO_4 , CaCl_2 , and FeCl_3 solutions/l of water. If dilution water is to be stored in the incubator, add the phosphate buffer just before using the dilution water.

b. Seeding: See ¶ 3*b* et seq, preceding. If the dilution water is seeded, use it the same day it is prepared.

c. Pretreatment:

1) Samples containing caustic alkalinity or acidity—Neutralize to about pH 7.0 with 1*N* H_2SO_4 or NaOH, using a pH meter or bromthymol blue as an outside indicator. The pH of the seeded dilution water should not be changed by the preparation of the lowest dilution of sample.

2) Samples containing residual chlorine compounds—If the samples stand for 1 to 2 hr, the residual chlorine often will be dissipated. Prepare BOD dilutions with properly seeded standard dilution water. Destroy higher chlorine residuals in neutralized samples by adding Na_2SO_3 . Determine the appropriate quantity of sodium sulfite solution on a 100- to 1,000-ml portion of the sample by adding 10 ml of 1+1 acetic acid or 1+50 H_2SO_4 , followed by 10 ml KI solution (10 g/100 ml) and titrating with 0.025*N* Na_2SO_3 solution to the starch-

iodide end point. Add to a volume of sample the quantity of Na_2SO_3 solution determined by the above test, mix, and after 10 to 20 min test a sample for residual chlorine to check the treatment. Prepare BOD dilutions with seeded standard dilution water.

3) Samples containing other toxic substances—Samples such as those from industrial wastes—for example, toxic metals derived from plating wastes—frequently require special study and treatment.

4) Samples supersaturated with DO—Samples containing more than 9 mg/l DO at 20 C may be encountered during winter months or in localities where algae are growing actively. To prevent loss of oxygen during incubation of these samples, reduce the DO to saturation by bringing the sample to about 20 C in a partly filled bottle and agitating it by vigorous shaking or by aerating with compressed air.

d. Dilution technic: Make several dilutions of the prepared sample to obtain the required depletions. The following dilutions are suggested: 0.1 to 1.0% for strong trade wastes, 1 to 5% for raw and settled sewage, 5 to 25% for oxidized effluents, and 25 to 100% for polluted river waters.

1) Carefully siphon standard dilution water, seeded if necessary, into a graduated cylinder of 1,000 to 2,000 ml capacity, filling the cylinder half full without entrainment of air. Add the quantity of carefully mixed sample to make the desired dilution and dilute to the appropriate level with dilution water. Mix well with a plunger-type mixing rod, avoiding entrainment of air. Siphon the mixed dilution into two BOD bottles, one for incubation and the other for de-

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termination of the initial DO in the mixture; stopper tightly and incubate for 5 days at 20 C. Water-seal the BOD bottles by inverting in a tray of water in the incubator or by using a special water-seal bottle. Prepare succeeding dilutions of lower concentration in the same manner or by adding dilution water to the unused portion of the preceding dilution.

2) The dilution technic may be greatly simplified when suitable amounts of sample are measured directly into bottles of known capacity with a large-tip volumetric pipet and the bottles are filled with sufficient dilution water to permit insertion of the stopper without leaving air bubbles. Make dilutions greater than 1:100 by diluting the waste in a volumetric flask before adding it to the incubation bottles for final dilution.

e. Determination of DO: If the sample represents 1% or more of the lowest BOD dilution, determine DO on the undiluted sample. This determination is usually omitted on sewage and settled effluents known to have a DO content of practically zero. *With samples having an immediate oxygen demand, use a calculated initial DO, inasmuch as such a demand represents a load on the receiving water.*

f. Incubation: Incubate the blank dilution water and the diluted samples for 5 days in the dark at 20 C. Then determine the DO in the incubated samples and the blank using the azide modification of the iodometric method or a membrane electrode. Unless the membrane electrode is used, use the alum flocculation method for incubated samples of muds and the copper sulfate-sulfamic acid method for activated sludges. In special cases, other modifications may

be necessary. Those dilutions showing a residual DO of at least 1 mg/l and a depletion of at least 2 mg/l are most reliable.

g. Seed correction: If the dilution water is seeded, determine the oxygen depletion of the seed by setting up a separate series of seed dilutions and selecting those resulting in 40 to 70% oxygen depletions in 5 days. Use one of these depletions to calculate the correction due to the small amount of seed in the dilution water. Do not use the seeded blank for seed correction because the 5-day seeded dilution water blank is subject to erratic oxidation due to the very high dilution of seed, which is not characteristic of the seeded sample.

b. Dilution water control: Fill two BOD bottles with unseeded dilution water. Stopper and water-seal one of these for incubation. Determine the DO before incubation in the other bottle. Use the DO results on these two bottles as a rough check on the quality of the unseeded dilution water. Do not use the depletion obtained as a blank correction; it should not be more than 0.2 mg/l and preferably not more than 0.1 mg/l.

i. Glucose-glutamic acid check: The BOD test is a bioassay procedure; consequently, the results obtained are influenced greatly by the presence of toxic substances or the use of a poor seeding material. Distilled waters frequently are contaminated with toxic substances—most often copper—and some sewage seeds are relatively inactive. The results obtained with such waters are always low.

The quality of the dilution water, the effectiveness of the seed, and the technic of the analyst should be checked periodically by using pure organic compounds

having known or determinable BOD. If a particular organic compound is known to be present in a given waste, it may well serve as a control on the seed used. For general BOD work, a mixture of glucose and glutamic acid (150 mg/l of each) has certain advantages. Glucose has an exceptionally high and variable oxidation rate with relatively simple seeds. When it is used with glutamic acid, the oxidation rate is stabilized and is similar to that obtained with many municipal wastes (0.16 to 0.19 exponential rate). In exceptional cases, a given component of a particular waste may be the best choice to test the efficacy of a particular seed.

To check the dilution water, the seed material, and the technic of the analyst, prepare a standard solution containing 150 mg/l each of reagent-grade glucose and glutamic acid that have been dried at 103 C for 1 hr. Pipet 5.0 ml of this solution into calibrated incubation bottles, fill with seeded dilution water, and incubate with seed control at 20 C for 5 days. On the basis of a mixed primary standard containing 150 mg/l each of glucose and glutamic acid, the 5-day BOD varies in magnitude according to the type of seed, and precision varies

with the quality of seed, as shown in Table 507:I.

Except with the oxidized river water and effluents, a low seed correction resulted in an appreciably higher value for the standard deviation. Check each seed source to determine the amount required to obtain optimum precision. If results differ appreciably from those given in Table 507:I after the seed source has been considered, the technic is questionable.

j. Immediate dissolved oxygen demand: Substances oxidizable by molecular oxygen, such as ferrous iron, sulfite, sulfide, and aldehyde, impose a load on the receiving water and must be taken into consideration. The total oxygen demand of such a substrate may be determined by using a calculated initial DO or by using the sum of the immediate dissolved oxygen demand (IDOD) and the 5-day BOD. Where a differentiation of the two components is desired, determine the IDOD. The IDOD does not necessarily represent the immediate oxidation by molecular DO but may represent an oxidation by the iodine liberated in the acidification step of the iodometric method.

TABLE 507:I. EFFECT OF SEED TYPE AND QUALITY ON BOD RESULTS

Type of Seed	5-day Seed Correction mg/l	Mean 5-day BOD mg/l	Standard Deviation mg/l
Settled fresh sewage	>0.6	218	±11
Settled stale sewage	>0.6	207	± 8
River water (4 sources)	0.05-0.22	224-242	±7-13
Activated sludge effluent	0.07-0.68	221	±13
Trickling filter effluent	0.2-0.4	225	± 8

The depletion of DO in a standard water dilution of the sample in 15 min has been arbitrarily selected as the IDOD. To determine the IDOD, separately measure the DO of the sample (which in most cases is zero) and the DO of the dilution water. Prepare an appropriate dilution of the sample and dilution water and determine the DO after 15 min. The calculated DO of the sample dilution minus the observed DO after 15 min is the IDOD, in milligrams per liter, of the sample dilution.

5. Calculation

a. Definitions:

D_0 = DO of original dilution water

D_1 = DO of diluted sample 15 min after preparation

D_2 = DO of diluted sample after incubation

S = DO of original undiluted sample

D_c = DO available in dilution at zero time
= $D_0 + SP$

p = decimal fraction of dilution water used

P = decimal fraction of sample used

B_1 = DO of dilution of seed control before incubation

B_2 = DO of dilution of seed control after incubation

f = ratio of seed in sample to seed in control
= $\frac{\% \text{ seed in } D_1}{\% \text{ seed in } B_1}$

Seed correction = $(B_1 - B_2)f$.

b. Biochemical oxygen demand:

When seeding is not required,

$$\text{mg/l BOD} = \frac{D_1 - D_2}{P}$$

When using seeded dilution water,

$$\text{mg/l BOD} = \frac{(D_1 - D_2) - (B_1 - B_2)f}{P}$$

Including IDOD if small or not determined,

$$\text{mg/l BOD} = \frac{D_c - D_1}{P}$$

c. Immediate dissolved oxygen demand:

$$\text{mg/l IDOD} = \frac{D_c - D_1}{P}$$

The DO determined on the unseeded dilution water after incubation is not used in the BOD calculations because this practice would overcorrect for the dilution water. In all the above calculations, corrections are not made for small losses of DO in the dilution water during incubation. If the dilution water is unsatisfactory, proper corrections are difficult and the results are questionable.

6. Precision and Accuracy

At present there is no standard against which the accuracy of the BOD test can be measured. To obtain inter-laboratory precision data, a glucose-glutamic acid mixture (¶4i preceding) with a theoretical oxygen demand value of 194 mg/l was analyzed by 73 participants, with each laboratory using its own seed material. The arithmetic mean of all results was 175 mg/l and the standard deviation of that mean was ± 26 mg/l (15%).

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pH

Method 150.1 (Electrometric)

STORET NO.

Determined on site 00400

Laboratory 00403

1. Scope and Application
 - 1.1 This method is applicable to drinking, surface, and saline waters, domestic and industrial wastes.
2. Summary of Method
 - 2.1 The pH of a sample is determined electrometrically using either a glass electrode in combination with a reference potential or a combination electrode.
3. Sample Handling and Preservation
 - 3.1 Samples should be analyzed as soon as possible preferably in the field at the time of sampling.
 - 3.2 High-purity waters and waters not at equilibrium with the atmosphere are subject to changes when exposed to the atmosphere, therefore the sample containers should be filled completely and kept sealed prior to analysis.
4. Interferences
 - 4.1 The glass electrode, in general, is not subject to solution interferences from color, turbidity, colloidal matter, oxidants, reductants or high salinity.
 - 4.2 Sodium error at pH levels greater than 10 can be reduced or eliminated by using a "low sodium error" electrode.
 - 4.3 Coatings of oily material or particulate matter can impair electrode response. These coatings can usually be removed by gentle wiping or detergent washing, followed by distilled water rinsing. An additional treatment with hydrochloric acid (1 + 9) may be necessary to remove any remaining film.
 - 4.4 Temperature effects on the electrometric measurement of pH arise from two sources. The first is caused by the change in electrode output at various temperatures. This interference can be controlled with instruments having temperature compensation or by calibrating the electrode-instrument system at the temperature of the samples. The second source is the change of pH inherent in the sample at various temperatures. This error is sample dependent and cannot be controlled, it should therefore be noted by reporting both the pH and temperature at the time of analysis.
5. Apparatus (ORION)
 - 5.1 pH Meter-laboratory or field model. A wide variety of instruments are commercially available with various specifications and optional equipment.

Approved for NPDES

Issued 1971

Editorial revision 1978

- 5.2 Glass electrode.
- 5.3 Reference electrode—a calomel, silver-silver chloride or other reference electrode of constant potential may be used.
NOTE 1: Combination electrodes incorporating both measuring and reference functions are convenient to use and are available with solid, gel type filling materials that require minimal maintenance.
- 5.4 Magnetic stirrer and Teflon-coated stirring bar.
- 5.5 Thermometer or temperature sensor for automatic compensation.
- 6. Reagents
 - 6.1 Primary standard buffer salts are available from the National Bureau of Standards and should be used in situations where extreme accuracy is necessary.
 - 6.1.1 Preparation of reference solutions from these salts require some special precautions and handling⁽¹⁾ such as low conductivity dilution water, drying ovens, and carbon dioxide free purge gas. These solutions should be replaced at least once each month.
 - 6.2 Secondary standard buffers may be prepared from NBS salts or purchased as a solution from commercial vendors. Use of these commercially available solutions, that have been validated by comparison to NBS standards, are recommended for routine use.
- 7. Calibration
 - 7.1 Because of the wide variety of pH meters and accessories, detailed operating procedures cannot be incorporated into this method. Each analyst must be acquainted with the operation of each system and familiar with all instrument functions. Special attention to care of the electrodes is recommended.
 - 7.2 Each instrument/electrode system must be calibrated at a minimum of two points that bracket the expected pH of the samples and are approximately three pH units or more apart.
 - 7.2.1 Various instrument designs may involve use of a “balance” or “standardize” dial and/or a slope adjustment as outlined in the manufacturer’s instructions. Repeat adjustments on successive portions of the two buffer solutions as outlined in procedure 8.2 until readings are within 0.05 pH units of the buffer solution value.
- 8. Procedure
 - 8.1 Standardize the meter and electrode system as outlined in Section 7.
 - 8.2 Place the sample or buffer solution in a clean glass beaker using a sufficient volume to cover the sensing elements of the electrodes and to give adequate clearance for the magnetic stirring bar.
 - 8.2.1 If field measurements are being made the electrodes may be immersed directly in the sample stream to an adequate depth and moved in a manner to insure sufficient sample movement across the electrode sensing element as indicated by drift free (<0.1 pH) readings.
 - 8.3 If the sample temperature differs by more than 2°C from the buffer solution the measured pH values must be corrected. Instruments are equipped with automatic or manual

⁽¹⁾National Bureau of Standards Special Publication 260.

compensators that electronically adjust for temperature differences. Refer to manufacturer's instructions.

- 8.4 After rinsing and gently wiping the electrodes, if necessary, immerse them into the sample beaker or sample stream and stir at a constant rate to provide homogeneity and suspension of solids. Rate of stirring should minimize the air transfer rate at the air water interface of the sample. Note and record sample pH and temperature. Repeat measurement on successive volumes of sample until values differ by less than 0.1 pH units. Two or three volume changes are usually sufficient.

9. Calculation

- 9.1 pH meters read directly in pH units. Report pH to the nearest 0.1 unit and temperature to the nearest °C.

10. Precision and Accuracy

- 10.1 Forty-four analysts in twenty laboratories analyzed six synthetic water samples containing exact increments of hydrogen-hydroxyl ions, with the following results:

pH Units	Standard Deviation pH Units	Accuracy as	
		Bias, %	Bias, pH Units
3.5	0.10	-0.29	-0.01
3.5	0.11	-0.00	
7.1	0.20	+1.01	+0.07
7.2	0.18	-0.03	-0.002
8.0	0.13	-0.12	-0.01
8.0	0.12	+0.16	+0.01

(FWPCA Method Study 1, Mineral and Physical Analyses)

- 10.2 In a single laboratory (EMSL), using surface water samples at an average pH of 7.7, the standard deviation was ± 0.1 .

Bibliography

1. Standard Methods for the Examination of Water and Wastewater, 14th Edition, p 460, (1975).
2. Annual Book of ASTM Standards, Part 31, "Water", Standard D1293-65, p 178 (1976).

IRON
Method 236.1 (Atomic Absorption, direct aspiration)

STORET NO. Total 01045

Dissolved 01046

Suspended 01044

Optimum Concentration Range: 0.3–5 mg/l using a wavelength of 248.3 nm

Sensitivity: 0.12 mg/l

Detection Limit: 0.03 mg/l

Preparation of Standard Solution

1. Stock Solution: Carefully weigh 1.000 g of pure iron wire (analytical reagent grade) and dissolve in 5 ml redistilled HNO_3 , warming if necessary. When solution is complete make up to 1 liter with deionized distilled water. 1 ml = 1 mg Fe (1000 mg/l).
2. Prepare dilutions of the stock solution to be used as calibration standards at the time of analysis. The calibration standards should be prepared using the same type of acid and at the same concentration as will result in the sample to be analyzed either directly or after processing.

Sample Preservation

1. For sample handling and preservation, see part 4.1 of the Atomic Absorption Methods section of this manual.

Sample Preparation

1. The procedures for preparation of the sample as given in parts 4.1.1 thru 4.1.4 of the Atomic Absorption Methods section of this manual have been found to be satisfactory.

Instrumental Parameters (General)

1. Iron hollow cathode lamp
2. Wavelength: 248.3 nm
3. Fuel: Acetylene
4. Oxidant: Air
5. Type of flame: Oxidizing

Analysis Procedure

1. For analysis procedure and calculation, see "Direct Aspiration", part 9.1 of the Atomic Absorption Methods section of this manual.

Approved for NPDES

Issued 1971

Editorial revision 1974 and 1978

Notes

1. The following lines may also be used:
248.8 nm Relative Sensitivity 2
271.9 nm Relative Sensitivity 4
302.1 nm Relative Sensitivity 5
252.7 nm Relative Sensitivity 6
372.0 nm Relative Sensitivity 10
2. Data to be reported into **STORET** must be reported as *ug*/1.
3. The 1,10-phenanthroline colorimetric method may also be used (Standard Methods, 14th Edition, p. 208).
4. For concentrations of iron below 0.05 mg/1, either the Special Extraction Procedure given in part 9.2 of the Atomic Absorption Methods section or the furnace procedure, Method 236.2, is recommended.

Precision and Accuracy

1. An interlaboratory study on trace metal analyses by atomic absorption was conducted by the Quality Assurance and Laboratory Evaluation Branch of EMSL. Six synthetic concentrates containing varying levels of aluminum, cadmium, chromium, copper, iron, manganese, lead and zinc were added to natural water samples. The statistical results for iron were as follows:

<u>Number Of Labs</u>	<u>True Values ug/liter</u>	<u>Mean Value ug/liter</u>	<u>Standard Deviation ug/liter</u>	<u>Accuracy as % Bias</u>
82	840	855	173	1.8
85	700	680	178	-2.8
78	350	348	131	-0.5
79	438	435	183	-0.7
57	24	58	69	141
54	10	48	69	382

NOTE 1: Chromic acid may be useful to remove organic deposits from glassware; however, the analyst should be cautioned that the glassware must be thoroughly rinsed with water to remove the last traces of chromium. This is especially important if chromium is to be included in the analytical scheme. A commercial product—NOCHROMIX—available from Godax Laboratories, 6 Varick St. New York, N.Y. 10013, may be used in place of chromic acid. [Chromic acid should not be used with plastic bottles.]

NOTE 2: If it can be documented through an active analytical quality control program using spiked samples, reagent and sample blanks, that certain steps in the cleaning procedure are not required for routine samples, those steps may be eliminated from the procedure.

Before collection of the sample a decision must be made as to the type of data desired, i.e., dissolved, suspended, total or total recoverable. For container preference, maximum holding time and sample preservation at time of collection see Table 1 in the front part of this manual. Drinking water samples containing suspended and settleable material should be prepared using the total recoverable metal procedure (section 4.1.4).

4.1.1 For the determination of dissolved constituents the sample must be filtered through a 0.45 μ membrane filter as soon as practical after collection. (Glass or plastic filtering apparatus using plain, non-grid marked, membrane filters are recommended to avoid possible contamination.) Use the first 50–100 ml to rinse the filter flask. Discard this portion and collect the required volume of filtrate. Acidify the filtrate with 1:1 redistilled HNO_3 to a pH of <2 . Normally, 3 ml of (1:1) acid per liter should be sufficient to preserve the sample (See Note 3). If hexavalent chromium is to be included in the analytical scheme, a portion of the filtrate should be transferred before acidification to a separate container and analyzed as soon as possible using Method 218.3. Analyses performed on a sample so treated shall be reported as “dissolved” concentrations.

NOTE 3: If a precipitate is formed upon acidification, the filtrate should be digested using 4.1.3. Also, it has been suggested (*International Biological Program, Symposium on Analytical Methods, Amsterdam, Oct. 1966*) that additional acid, as much as 25 ml of conc. HCl /liter, may be required to stabilize certain types of highly buffered samples if they are to be stored for any length of time. Therefore, special precautions should be observed for preservation and storage of unusual samples intended for metal analysis.

4.1.2 For the determination of suspended metals a representative volume of unpreserved sample must be filtered through a 0.45 μ membrane filter. When considerable suspended material is present, as little as 100 ml of a well mixed sample is filtered. Record the volume filtered and transfer the membrane filter containing the insoluble material to a 250 ml Griffin beaker and add 3 ml conc. redistilled HNO_3 . Cover the beaker with a watch glass and heat gently. The warm acid will soon dissolve the membrane. Increase the temperature of the hot plate and digest the material. When the acid has nearly evaporated, cool the beaker and watch glass and add another 3 ml of conc. redistilled HNO_3 . Cover and continue heating until

the digestion is complete, generally indicated by a light colored digestate. Evaporate to near dryness (DO NOT BAKE), add 5 ml distilled HCl (1:1) and warm the beaker gently to dissolve any soluble material. (If the sample is to be analyzed by the furnace procedure, 1 ml of 1:1 distilled HNO₃ per 100 ml dilution should be substituted for the distilled 1:1 HCl.) Wash down the watch glass and beaker walls with deionized distilled water and filter the sample to remove silicates and other insoluble material that could clog the atomizer. Adjust the volume to some predetermined value based on the expected concentrations of metals present. This volume will vary depending on the metal to be determined. The sample is now ready for analysis. Concentrations so determined shall be reported as "suspended" (See Note 4.)

NOTE 4: Certain metals such as antimony arsenic, gold, iridium, mercury, osmium, palladium, platinum, rhenium, rhodium, ruthenium, selenium, silver, thallium, tin and titanium require modification of the digestion procedure and the individual sheets for these metals should be consulted.

- 4.1.3 For the determination of total metals the sample is acidified with 1:1 redistilled HNO₃ to a pH of less than 2 at the time of collection. The sample is not filtered before processing. Choose a volume of sample appropriate for the expected level of metals. If much suspended material is present, as little as 50–100 ml of well mixed sample will most probably be sufficient. (The sample volume required may also vary proportionally with the number of metals to be determined.)

Transfer a representative aliquot of the well mixed sample to a Griffin beaker and add 3 ml of conc. redistilled HNO₃. Place the beaker on a hot plate and evaporate to near dryness cautiously, making certain that the sample does not boil. (DO NOT BAKE.) Cool the beaker and add another 3 ml portion of conc. redistilled HNO₃. Cover the beaker with a watch glass and return to the hot plate. Increase the temperature of the hot plate so that a gentle reflux action occurs. Continue heating, adding additional acid as necessary, until the digestion is complete (generally indicated when the digestate is light in color or does not change in appearance with continued refluxing). Again, evaporate to near dryness and cool the beaker. Add a small quantity of redistilled 1:1 HCl (5 ml/100 ml of final solution) and warm the beaker to dissolve any precipitate or residue resulting from evaporation. (If the sample is to be analyzed by the furnace procedure, substitute distilled HNO₃ for 1:1 HCl so that the final dilution contains 0.5% (v/v) HNO₃.) Wash down the beaker walls and watch glass with distilled water and filter the sample to remove silicates and other insoluble material that could clog the atomizer. Adjust the volume to some predetermined value based on the expected metal concentrations. The sample is now ready for analysis. Concentrations so determined shall be reported as "total" (see Note 4).

- 4.1.4 To determine total recoverable metals, acidify the entire sample at the time of collection with conc. redistilled HNO₃, 5 ml/l. At the time of analysis a 100 ml aliquot of well mixed sample is transferred to a beaker or flask. Five ml of distilled HCl (1:1) is added and the sample heated on a steam bath or hot plate until the

RESIDUE, FILTERABLE

Method 160.1 (Gravimetric, Dried at 180°C)

STORET NO. 70300

1. Scope and Application
 - 1.1 This method is applicable to drinking, surface, and saline waters, domestic and industrial wastes.
 - 1.2 The practical range of the determination is 10 mg/l to 20,000 mg/l.
2. Summary of Method
 - 2.1 A well-mixed sample is filtered through a standard glass fiber filter. The filtrate is evaporated and dried to constant weight at 180°C.
 - 2.2 If Residue, Non-Filterable is being determined, the filtrate from that method may be used for Residue, Filterable.
3. Definitions
 - 3.1 Filterable residue is defined as those solids capable of passing through a glass fiber filter and dried to constant weight at 180°C.
4. Sample Handling and Preservation
 - 4.1 Preservation of the sample is not practical; analysis should begin as soon as possible. Refrigeration or icing to 4°C, to minimize microbiological decomposition of solids, is recommended.
5. Interferences
 - 5.1 Highly mineralized waters containing significant concentrations of calcium, magnesium, chloride and/or sulfate may be hygroscopic and will require prolonged drying, desiccation and rapid weighing.
 - 5.2 Samples containing high concentrations of bicarbonate will require careful and possibly prolonged drying at 180°C to insure that all the bicarbonate is converted to carbonate.
 - 5.3 Too much residue in the evaporating dish will crust over and entrap water that will not be driven off during drying. Total residue should be limited to about 200 mg.
6. Apparatus
 - 6.1 Glass fiber filter discs, 4.7 cm or 2.1 cm, without organic binder, Reeve Angel type 934-AH, Gelman type A/E, or equivalent.
 - 6.2 Filter holder, membrane filter funnel or Gooch crucible adapter.
 - 6.3 Suction flask, 500 ml.
 - 6.4 Gooch crucibles, 25 ml (if 2.1 cm filter is used).
 - 6.5 Evaporating dishes, porcelain, 100 ml volume. (Vycor or platinum dishes may be substituted).
 - 6.6 Steam bath.
 - 6.7 Drying oven, 180°C \pm 2°C.
 - 6.8 Desiccator.

Approved for NPDES

Issued 1971

- 6.9 Analytical balance, capable of weighing to 0.1 mg.
7. Procedure
- 7.1 Preparation of glass fiber filter disc: Place the disc on the membrane filter apparatus or insert into bottom of a suitable Gooch crucible. While vacuum is applied, wash the disc with three successive 20 ml volumes of distilled water. Remove all traces of water by continuing to apply vacuum after water has passed through. Discard washings.
- 7.2 Preparation of evaporating dishes: If Volatile Residue is also to be measured heat the clean dish to $550 \pm 50^{\circ}\text{C}$ for one hour in a muffle furnace. If only Filterable Residue is to be measured heat the clean dish to $180 \pm 2^{\circ}\text{C}$ for one hour. Cool in desiccator and store until needed. Weigh immediately before use.
- 7.3 Assemble the filtering apparatus and begin suction. Shake the sample vigorously and rapidly transfer 100 ml to the funnel by means of a 100 ml graduated cylinder. If total filterable residue is low, a larger volume may be filtered.
- 7.4 Filter the sample through the glass fiber filter, rinse with three 10 ml portions of distilled water and continue to apply vacuum for about 3 minutes after filtration is complete to remove as much water as possible.
- 7.5 Transfer 100 ml (or a larger volume) of the filtrate to a weighed evaporating dish and evaporate to dryness on a steam bath.
- 7.6 Dry the evaporated sample for at least one hour at $180 \pm 2^{\circ}\text{C}$. Cool in a desiccator and weigh. Repeat the drying cycle until a constant weight is obtained or until weight loss is less than 0.5 mg.
8. Calculation
- 8.1 Calculate filterable residue as follows:

$$\text{Filterable residue, mg/l} = \frac{(A - B) \times 1,000}{C}$$

where:

A = weight of dried residue + dish in mg

B = weight of dish in mg

C = volume of sample used in ml

9. Precision and Accuracy
- 9.1 Precision and accuracy are not available at this time.

Bibliography

1. Standard Methods for the Examination of Water and Wastewater, 14th Edition, p 92, Method 208B, (1975).

The UniVer Hardness Test

is used for hardness titrations of samples containing large amounts of interfering substances.

UniVer I contains a large proportion of sodium cyanide and hence it is poisonous. It will eliminate interferences from large amounts of copper, iron, aluminum, cobalt, nickel, and other metals. It is primarily used for special industrial analyses, such as the determination of hardness in sugar beet juice.

UniVer II contains a sodium carbonate type buffer and an inhibitor to prevent interference from copper. Iron interferes seriously. The powder is non-poisonous and very stable. Neither powder is suitable for trace analysis. Powdered Buffer-Indicators for hardness may give a reagent blank of several ppm.

Procedure

1. Measure a 50.0 ml water sample and pour it into a 250 ml flask or beaker.

2. Add the contents of one Powder Pillow of UniVer I or UniVer II (or a 1 gram measuring spoonful of UniVer I or UniVer II powder).

3. Titrate with Standard TitraVer solution until the solution changes from red to pure blue.

4. The total hardness as ppm Calcium Carbonate is calculated by multiplying the milliliters of the TitraVer solution used in the titration by 20.

Reagents and Apparatus

UniVer® I, No. 206	lb.	\$ 2.25
UniVer® II, No. 278	lb.	2.25
Measuring Spoon, 1.0 gram, No. 510	each	.25
UniVer® I Powder Pillows, 1.0 gram, No. 849	100	2.30
UniVer® II Powder Pillows, 1.0 gram, No. 850	100	2.35
TitraVer®, Standard Solution, No. 205	quart	1.60
HexaVer®, Standard Solution, No. 740	quart	2.25
Buret, Automatic, 25 ml, No. 504	each	12.15
Cylinder, Graduated, 50 ml, No. 508	each	3.26
Flask, Erlenmeyer, 250 ml, No. 505	each	.53
Standard Calcium Chloride Solution, 1 ml = 1 mg CaCO ₃ , No. 121	pint	1.40

Preparation of Reagents

The competent chemist can prepare his own TitraVer or HexaVer Solutions and/or his own Standard CaCl₂ Solution.

Procedure for preparation of TitraVer or HexaVer

1. Weigh 4 grams of TitraVer powder or HexaVer powder and dissolve it in 750 ml of water.

2. Using this solution, titrate a 25.0 ml sample of standard calcium chloride solution (Cat. No. 121), using the ManVer procedure above for the determination of total hardness in water.

3. From this titration calculate the strength of the TitraVer Solution or HexaVer Solution and calculate the volume to which the TitraVer Solution or HexaVer Solution should be diluted to make it 1 ml = 1 mg. calcium carbonate in strength.

Procedure for preparation of Standard CaCl₂ Solution

1. Dissolve 1.000 gram of dry primary standard calcium carbonate in a little dilute hydrochloric acid.

2. Dilute to exactly 1 liter. One ml of this solution is equivalent to 1.0 mg. calcium carbonate.

ALKALINITY

Method 310.1 (Titrimetric, pH 4.5)

STORET NO. 00410

1. Scope and Application
 - 1.1 This method is applicable to drinking, surface, and saline waters, domestic and industrial wastes.
 - 1.2 The method is suitable for all concentration ranges of alkalinity; however, appropriate aliquots should be used to avoid a titration volume greater than 50 ml.
 - 1.3 Automated titrimetric analysis is equivalent.
2. Summary of Method
 - 2.1 An unaltered sample is titrated to an electrometrically determined end point of pH 4.5. The sample must not be filtered, diluted, concentrated, or altered in any way.
3. Comments
 - 3.1 The sample should be refrigerated at 4°C and run as soon as practical. Do not open sample bottle before analysis.
 - 3.2 Substances, such as salts of weak organic and inorganic acids present in large amounts, may cause interference in the electrometric pH measurements.
 - 3.3 For samples having high concentrations of mineral acids, such as mine wastes and associated receiving waters, titrate to an electrometric endpoint of pH 3.9, using the procedure in:
Annual Book of ASTM Standards, Part 31, "Water", p 115, D-1067, Method D, (1976).
 - 3.4 Oil and grease, by coating the pH electrode, may also interfere, causing sluggish response.
4. Apparatus
 - 4.1 pH meter or electrically operated titrator that uses a glass electrode and can be read to 0.05 pH units. Standardize and calibrate according to manufacturer's instructions. If automatic temperature compensation is not provided, make titration at 25 ± 2° C.
 - 4.2 Use an appropriate sized vessel to keep the air space above the solution at a minimum. Use a rubber stopper fitted with holes for the glass electrode, reference electrode (or combination electrode) and buret.
 - 4.3 Magnetic stirrer, pipets, flasks and other standard laboratory equipment.
 - 4.4 Burets, Pyrex 50, 25 and 10 ml.
5. Reagents
 - 5.1 Sodium carbonate solution, approximately 0.05 N: Place 2.5 ± 0.2 g (to nearest mg) Na₂CO₃ (dried at 250°C for 4 hours and cooled in desiccator) into a 1 liter volumetric flask and dilute to the mark.

Approved for NPDES

Issued 1971

Editorial revision 1978

- 5.2 Standard acid (sulfuric or hydrochloric), 0.1 N: Dilute 3.0 ml conc H_2SO_4 or 8.3 ml conc HCl to 1 liter with distilled water. Standardize versus 40.0 ml of 0.05 N Na_2CO_3 solution with about 60 ml distilled water by titrating potentiometrically to pH of about 5. Lift electrode and rinse into beaker. Boil solution gently for 3–5 minutes under a watch glass cover. Cool to room temperature. Rinse cover glass into beaker. Continue titration to the pH inflection point. Calculate normality using:

$$N = \frac{A \times B}{53.00 \times C}$$

where:

A = g Na_2CO_3 weighed into 1 liter

B = ml Na_2CO_3 solution

C = ml acid used to inflection point

- 5.3 Standard acid (sulfuric or hydrochloric), 0.02 N: Dilute 200.0 ml of 0.1000 N standard acid to 1 liter with distilled water. Standardize by potentiometric titration of 15.0 ml 0.05 N Na_2CO_3 solution as above.

6. Procedure

6.1 Sample size

- 6.1.1 Use a sufficiently large volume of titrant (> 20 ml in a 50 ml buret) to obtain good precision while keeping volume low enough to permit sharp end point.
- 6.1.2 For < 1000 mg CaCO_3 /l use 0.02 N titrant
- 6.1.3 For > 1000 mg CaCO_3 /l use 0.1 N titrant
- 6.1.4 A preliminary titration is helpful.

6.2 Potentiometric titration

- 6.2.1 Place sample in flask by pipetting with pipet tip near bottom of flask
- 6.2.2 Measure pH of sample
- 6.2.3 Add standard acid (5.2 or 5.3), being careful to stir thoroughly but gently to allow needle to obtain equilibrium.
- 6.2.4 Titrate to pH 4.5. Record volume of titrant.

6.3 Potentiometric titration of low alkalinity

- 6.3.1 For alkalinity of < 20 mg/l titrate 100–200 ml as above (6.2) using a 10 ml microburet and 0.02 N acid solution (5.3).
- 6.3.2 Stop titration at pH in range of 4.3–4.7, record volume and exact pH. Very carefully add titrant to lower pH exactly 0.3 pH units and record volume.

7. Calculations

7.1 Potentiometric titration to pH 4.5

$$\text{Alkalinity, mg/l CaCO}_3 = \frac{A \times N \times 50,000}{\text{ml of sample}}$$

where:

A = ml standard acid

N = normality standard acid

7.2 Potentiometric titration of low alkalinity:

$$\text{Total alkalinity, mg/l CaCO}_3 = \frac{(2B - C) \times N \times 50,000}{\text{ml of sample}}$$

where:

B = ml titrant to first recorded pH

C = total ml titrant to reach pH 0.3 units lower

N = normality of acid

8. Precision and Accuracy

8.1 Forty analysts in seventeen laboratories analyzed synthetic water samples containing increments of bicarbonate, with the following results:

<u>Increment as</u> <u>Alkalinity</u> <u>mg/liter, CaCO₃</u>	<u>Precision as</u> <u>Standard Deviation</u> <u>mg/liter, CaCO₃</u>	<u>Bias,</u> <u>%</u>	<u>Accuracy as</u> <u>Bias,</u> <u>mg/l, CaCO₃</u>
8	1.27	+ 10.61	+0.85
9	1.14	+22.29	+2.0
113	5.28	- 8.19	-9.3
119	5.36	- 7.42	-8.8

(FWPCA Method Study 1, Mineral and Physical Analyses)

8.2 In a single laboratory (EMSL) using surface water samples at an average concentration of 122 mg CaCO₃/l, the standard deviation was ±3.

Bibliography

1. Standard Methods for the Examination of Water and Wastewater, 14th Edition, p 278, Method 403, (1975).
2. Annual Book of ASTM Standards, Part 31, "Water", p 113, D-1067, Method B, (1976).

PART III. ANALYTICAL METHODOLOGY

Section B Total Coliform Methods

This section describes the enumerative techniques for total coliform bacteria in water and wastewater. The method chosen depends upon the characteristics of the sample. The Section is divided as follows:

1. Definition of the Coliform Group
2. Single-Step, Two-Step and Delayed-Incubation Membrane Filter (MF) Methods
3. Verification
4. Most Probable Number (MPN) Method
5. Differentiation of the Coliform Group by Further Biochemical Tests

1. Definition of the Coliform Group

The coliform or total coliform group includes all of the aerobic and facultative anaerobic, gram-negative, nonspore-forming, rod-shaped bacteria that ferment lactose in 24-48 hours at 35 C. The definition includes the genera: *Escherichia*, *Citrobacter*, *Enterobacter*, and *Klebsiella*.

2. Single-Step, Two-Step and Delayed-Incubation Membrane Filter Methods

2.1 Summary: An appropriate volume of a water sample or its dilution is passed through a membrane filter that retains the bacteria present in the sample.

In the single-step procedure the filter retaining the microorganisms is placed on M-Endo agar, LES M-Endo agar or on an absorbent pad saturated with M-Endo broth in a petri dish. The test is incubated at 35 C for 24 hours.

In the two-step enrichment procedure the filter retaining the microorganisms is placed on an absorbent pad saturated with lauryl tryptose (lauryl sulfate) broth. After incubation for 2 hours at 35 C, the filter is transferred to an absorbent pad saturated with M-Endo broth, M-Endo agar, or LES M-Endo agar, and incubated for an additional 20-22 hours at 35 C. The sheen colonies are counted under low magnification and the numbers of total coliforms are reported per 100 ml of original sample.

In the delayed-incubation procedure, the filter retaining the microorganisms is placed on an absorbent pad saturated with M-Endo preservative medium in a tight-lidded petri dish and transported from field site to the laboratory. In the laboratory, the filter is transferred to M-Endo growth medium and incubated at 35 C for 24 hours. Sheen colonies are counted as total coliforms per 100 ml.

2.2 Scope and Application: The total coliform test can be used for any type of water or wastewater, but since the development of the fecal coliform procedure there has been increasing use of this more specific test as an indicator of fecal pollution. However, the total coliform test remains the primary indicator of bacteriological quality for potable water, distribution system waters, and public water supplies because a broader measure of pollution is desired for these waters. It is also a useful measure in shellfish-raising waters.

Although the majority of water and wastewater samples can be examined for total coliforms by the single-step MF procedure, coliforms may be suppressed by high background organisms, and potable water samples may require the two-step method.

If the membrane filtration method is used to measure total coliforms in chlorinated secondary or tertiary sewage effluents the two-step enrichment procedure is required. However, it may be necessary to use the MPN method because of high solids in the wastes or toxicity from an industrial waste (see Part II-D, this Manual).

The delayed-incubation MF method is useful in survey monitoring or emergency situations when the single step coliform test cannot be performed at the sample site, or when time and temperature limits for sample storage cannot be met. The method eliminates field processing and equipment needs. Also, examination at a central laboratory permits confirmation and biochemical identification of the organisms as necessary. Consistent results have been obtained with this method using water samples from a variety of sources (1, 2). The applicability of this method for a specific water source must be determined in preliminary studies by comparison with the standard MF method.

2.3 Apparatus and Materials

2.3.1 Water jacket, air, or heat sink incubator that maintains 35 ± 0.5 C. Temperature

is checked against an NBS certified thermometer or equivalent. Incubator must have humidity control if loose-lidded petri dishes are used. See Part II-B, 1.2.

2.3.2 A binocular (dissection) microscope, with magnification of 10 or 15 \times , and a daylight type fluorescent lamp angled to give maximum sheen appearance.

2.3.3 Hand tally.

2.3.4 Pipet container of stainless steel, aluminum or pyrex glass for glass pipets.

2.3.5 Sterile 50–100 ml graduated cylinders covered with aluminum foil or kraft paper,

2.3.6 Sterile, unassembled membrane filtration units (filter base and funnel), glass, plastic or stainless steel, wrapped with aluminum foil or kraft paper. Portable field filtration units are available.

2.3.7 Vacuum source.

2.3.8 Vacuum filter flask with appropriate tubing. Filter manifolds which hold a number of filter bases can also be used.

2.3.9 Ultraviolet sterilizer for MF filtration units (optional).

2.3.10 Safety trap flask between the filter flask and the vacuum source.

2.3.11 Forceps with smooth tips.

2.3.12 Methanol or ethanol, 95%, in small vial, for flaming forceps.

2.3.13 Bunsen/Fisher burner or electric incinerator.

2.3.14 Sterile TD bacteriological or Mohr pipets, glass or plastic, of appropriate size.

2.3.15 Sterile petri dishes with tight-fitting lids, 50 \times 12 mm or loose-fitting lids 60 \times 15 mm, glass or plastic.

2.3.16 Dilution bottles (milk dilution), pyrex, marked at 99 ml volume, screw cap with neoprene rubber liner.

2.3.17 Membrane filters, white, grid-marked, 47 mm diameter, with $0.45\ \mu\text{m} \pm 0.02\ \mu\text{m}$ pore size, or other pore size, as recommended by manufacturer for water analyses.

2.3.18 Absorbent pads.

2.3.19 Inoculation loops, at least 3 mm diameter, or needles, nichrome or platinum wire, 26 B&S gauge, in suitable holder.

2.3.20 Disposable applicator sticks or plastic loops as alternatives to inoculation loops.

2.3.21 Shipping tubes, labels, and packing materials for mailing delayed incubation plates.

2.4 Media: Media are prepared in pre-sterilized erlenmeyer flasks with metal caps, aluminum foil covers, or screw caps.

2.4.1 M-Endo broth or agar (See Part II-B, 5.2.2).

2.4.2 LES M-Endo agar (See Part II-B, 5.2.4).

2.4.3 Lauryl tryptose broth (See Part II-B, 5.3.1).

2.4.4 Brilliant green lactose bile broth (See Part II-B, 5.3.2).

2.4.5 M-Endo holding medium (See Part II-B, 5.2.3).

2.4.6 Sodium benzoate, U.S.P., for use in the delayed incubation procedure (See Part II-B, 5.2.3).

2.4.7 Cycloheximide (Actidione - Upjohn, Kalamazoo, MI) for use as antifungal agent in delayed incubation procedure (See Part II-B, 5.2.3).

2.5 Dilution Water (See Part II-B, 7 for preparation).

2.5.1 Sterile dilution water dispensed in 99 ± 2 ml amounts in screw-capped dilution bottles.

2.5.2 Sterile dilution water prepared in 1 liter or larger volumes for wetting membranes before addition of small sample volumes and for rinsing the funnel after sample filtration.

2.6 Procedure: Refer to the general procedure in Part II-C for more complete details.

2.6.1 Single-Step Procedure

(a) Prepare the M-Endo broth, M-Endo agar or LES M-Endo agar as directed in Part II-B.

(b) Place one sterile absorbent pad in the bottom half of each petri dish. Pipet 1.8–2.0 ml M-Endo broth onto the pad to saturate it. Pour off excess broth. Alternatively, pipet 5–6 ml of melted agar into each dish (2–3 mm) and allow to harden before use. Mark dishes and bench forms with sample identities and volumes.

(c) Place a sterile membrane filter on the filter base, grid-side up and attach the funnel to the base of the filter unit; the membrane filter is now held between the funnel and the base.

(d) Shake the sample bottle vigorously about 25 times and measure the desired volume of sample into the funnel. Select sample volumes based on previous knowledge to produce membrane filters with 20–80 coliform colonies. See Table II-C-1. If sample volume is < 10 ml, add 10 ml of sterile dilution water to the filter before adding sample.

It is desirable to filter the largest possible sample volumes for greatest accuracy. However, if past analyses of specific samples have resulted in confluent growth, "too numerous to count" membranes, or lack of sheen from excessive turbidity, additional samples should be collected and filtration volumes adjusted to provide isolated colonies from smaller volumes. See 2.7.2 in this Section for details on adjusting sample volumes for potable waters.

The suggested method for measuring sample volumes is described in Part II-C, 3.4.6.

(e) Filter sample and rinse the sides of the funnel at least twice with 20–30 ml of sterile dilution water. Turn off the vacuum and remove the funnel from the filter base. Aseptically remove the membrane filter from the filter base and place grid-side up on the agar or pad.

(f) Filter samples in order of increasing sample volume, filter potable waters first.

(g) If M-Endo broth is used, place the filter on an absorbent pad saturated with the broth. Reseat the membrane, if air bubbles occur, as evidenced by non-wetted areas on the membrane. Invert dish and incubate for 24 ± 2 hours at 35 ± 0.5 C in an atmosphere with near saturated humidity.

(h) If M-Endo agar or LES M-Endo agar is used, place the inoculated filter directly on the agar surface. Reseat the membrane if bubbles occur. Invert the dish and incubate for 24 ± 2 hours at 35 ± 0.5 C in an atmosphere with near saturated humidity.

(i) If tight-lidded dishes are used, there is no requirement for near-saturated humidity.

(j) After incubation remove the dishes from the incubator and examine for sheen colonies.

(k) Proceed to 2.7 for Counting and Recording Colonies.

2.6.2 Two-Step Enrichment Procedure

(a) Place a sterile absorbent pad in the top of each petri dish.

(b) Prepare lauryl tryptose broth as directed in Part II-B. Pipet 1.8–2.0 ml lauryl tryptose broth onto the pad to saturate it. Pour off excess broth.

(c) Place a sterile membrane filter on the filter holder, grid-side up and attach the funnel to the base of the filter unit; the membrane

filter is now held between the funnel and the base.

(d) Shake the sample bottle vigorously about 25 times to obtain uniform distribution of bacteria. Select sample volumes based on previous knowledge to produce membrane filters with 20–80 coliform colonies. See Table II-C-1. If sample volume is < 10 ml, add 10 ml of sterile dilution water to filter before adding sample.

(e) Filter samples in order of increasing sample volume, rinsing with sterile buffered dilution water between filtrations. The methods of measurement and dispensation of the sample into the funnel are given in Part II-C, 3.4.6.

(f) Turn on the vacuum to filter the sample through the membrane, rinse the sides of the funnel at least twice with 20–30 ml of sterile dilution water. Turn off vacuum and remove funnel from base.

(g) Remove the membrane filter aseptically from the filter base and place grid-side up on the pad in the top of the petri dish. Reseat MF if air bubbles are observed.

(h) Incubate the filter in the petri dish without inverting for 1 1/2 – 2 hours at 35 ± 0.5 C in an atmosphere of near saturated humidity. This completes the first step in the Two-Step Enrichment Procedure.

(i) Prepare M-Endo broth, M-Endo agar, or LES M-Endo agar as directed in Part II-B.

If M-Endo broth is used, place a new sterile absorbent pad in the bottom half of the dish and saturate with 1.8–2.0 ml of the M-Endo broth. Transfer the filter to the new pad. Reseat MF if air bubbles are observed. Remove the used pad and discard.

If M-Endo or LES M-Endo agar is used, pour 5–6 ml of agar into the bottom of each petri dish and allow to solidify. The agar medium can be refrigerated for up to two weeks.

(j) Transfer the filter from the lauryl tryptose broth onto the Endo medium. Reseat if air bubbles are observed.

(k) Incubate dishes in an inverted position for an additional 20–22 hours at 35 ± 0.5 C. This completes the second step in the Two-Step Enrichment Procedure.

(l) Proceed to 2.7 Counting and Recording.

2.6.3 Delayed Incubation Procedure

(a) Prepare the M-Endo Holding Medium or LES Holding Medium as outlined in Part II-B, 5.2.3 or 5.2.5. Saturate the sterile absorbent pads with about 2.0 ml of holding broth. Pour off excess broth. Mark dishes and bench forms with sample identity and volumes.

(b) Using sterile forceps place a membrane filter on the filter base grid side up.

(c) Attach the funnel to the base of the filter unit; the membrane filter is now held between the funnel and base.

(d) Shake the sample vigorously about 25 times and measure into the funnel with the vacuum off. If the sample is < 10 ml, add 10 ml of sterile dilution water to the membrane filter before adding the sample.

(1) Select sample volumes based on previous knowledge to produce counts of 20–80 coliform colonies. See Table II-C-1.

(2) Follow the methods for sample measurement and dispensation given in Part II-C, 3.4.6

(e) Filter the sample through the membrane and rinse the sides of the funnel walls at least twice with 20–30 ml of sterile dilution water.

(f) Turn off the vacuum and remove the funnel from the base of the filter unit.

(g) Aseptically remove the membrane filter from the filter base and place grid side up on

an absorbent pad saturated with M-Endo Holding Medium or LES Holding Medium.

(h) Place the culture dish in shipping container and send to the examining laboratory. Coliform bacteria can be held on the holding medium for up to 72 hours with little effect on the final counts. The holding period should be kept to a minimum.

(i) At the examining laboratory remove the membrane from the holding medium, place it in another dish containing M-Endo broth or agar medium, and complete testing for coliforms as described above under 2.6.1.

2.7 Counting and Recording Colonies:

After incubation, count colonies on those membrane filters containing 20–80 golden-green metallic surface sheen colonies and less than 200 total bacterial colonies. A binocular (dissection) microscope with a magnification of 10 or 15 \times is recommended. Count the colonies according to the general directions given in Part II-C, 3.5.

2.7.1 The following general rules are used in calculating the total coliform count per 100 ml of sample. Specific rules for analysis and counting of water supply samples are given in 2.7.2.

(a) Countable Membranes with 20–80 Sheen Colonies, and Less Than 200 Total Bacterial Colonies: Select the plate counts to be used according to the rules given in Part II-C, 3.6, and calculate the final value using the formula.

Total Coliforms/100 ml:

No. of Total Coliform Colonies Counted

Volume in ml of Sample Filtered

$\times 100$

(b) Counts Greater Than the Upper Limit of 80 Colonies: All colony counts are above the recommended limits. For example, sample volumes of 1, 0.3, and 0.01 ml are filtered to

produce total coliform colony counts of TNC, 150, and 110 colonies.

Use the count from the smallest filtration volume and report as a greater than count/100 ml. In the example above:

$$\frac{110}{0.01} \times 100 = 1,100,000$$

or > 1,100,000 coliforms/100 ml.

(c) Membranes with More Than 200 Total Colonies (Coliforms plus Non-coliforms).

(1) Estimate sheen colonies if possible, calculate total coliform density as in (a) above.

Report as: Estimated Count/100 ml.

(2) If estimate of sheen colonies is not possible, report count as Too Numerous to Count (TNTC).

(d) Membranes with Confluent Growth

Report as: Confluent Growth and specify the presence or absence of sheen.

2.7.2 Special Rules for Potable Waters

(a) Countable Membranes with 0-80 Sheen Colonies, and Less than 200 Total Colonies

Count the sheen colonies per volume filtered. Calculate and report the number of Total Coliforms/100 ml.

(b) Uncountable Membranes for Potable Water Samples

If 100 ml portions of potable water samples cannot be tested because of high background counts or confluency, multiple volumes of less than 100 ml can be filtered. For example, if 60 colonies appear on the surface of one membrane through which a 50 ml portion of the sample was passed, and 50 colonies on a second membrane through which a second 50 ml portion of the sample was passed, the colonies are totaled and reported as 110 total coliforms per 100 ml.

If filtration of multiple volumes of less than 100 ml still results in confluency or high background count, the coliforms may be present but suppressed. These samples should be analyzed by the MPN Test. This MPN check should be made on at least one sample for each problem water once every three months.

(c) Membranes with Confluent Growth

For potable water samples, confluence requires resampling and retesting.

(d) Verification. Because unsatisfactory samples from public water supplies containing 5 or more coliform colonies must be verified, at least 5 colonies need to be verified for each positive sample. Reported counts are adjusted based on verification.

(e) Quality control procedures are specified by EPA under the law, and described in Appendix C in this Manual.

2.7.3 Reporting Results: Report total coliform densities per 100 ml of sample. See Figure II-C-3 for an example of a bench form for reporting results. A discussion on significant figures is given in Part II-C, 2.8.

2.8 Precision and Accuracy: There are no established precision and accuracy data available at this time.

3. Verification

Verification of total coliform colonies from M-Endo type media validates sheen as evidence of coliforms. Verification of representative numbers of colonies may be required in evidence gathering or for quality control procedures. The verification procedure follows:

3.1 Using a sterile inoculating needle, pick growth from the centers of at least 10 well-isolated sheen colonies (5 sheen colonies per plate for potable waters). Inoculate each into a tube of lauryl tryptose broth and incubate 24-48 hours at 35 C ± 0.5 C. Do not transfer exclusively into brilliant green bile lactose broth. However, colonies may be transferred to LTB and BGLB simultaneously.

3.2 At the 24 and 48 hour readings, confirm gas-positive lauryl tryptose broth tubes by inoculating a loopful of growth into brilliant green lactose bile broth and incubate for 24-48 hours at 35 ± 0.5 C. Cultures that are positive in BGLB are interpreted as verified coliform colonies (see Figure III-B-1).

3.3 If questionable sheen occurs, the worker should also verify these colonies.

4. Most Probable Number (MPN) Method

4.1 Summary: This method detects and estimates the total coliforms in water samples by the multiple fermentation tube technique. The method has three stages: the Presumptive, the Confirmed, and the Completed Tests. In the Presumptive Test, a series of lauryl tryptose broth fermentation tubes are inoculated with decimal dilutions of the sample. The formation of gas at 35 C within 48 hours constitutes a positive Presumptive Test for members of the total coliform group. However, the MPN must be carried through the Confirmed Test for valid results. In this test, inocula from positive Presumptive tubes are transferred to tubes of brilliant green lactose bile (BGLB) broth. The BGLB medium contains selective and inhibitive agents to suppress the growth of all non-coliform organisms. Gas production after incubation for 24 or 48 hours at 35 C constitutes a positive Confirmed Test and is the point at which most MPN tests are terminated. The Completed Test begins with streaking inoculum from the positive BGLB tubes onto EMB plates and incubating the plates for 24 hours at 35 C. Typical and atypical colonies are transferred into lauryl tryptose broth fermentation tubes and onto nutrient agar slants. Gas formation in the fermentation tubes and presence of gram-negative rods constitute a positive Completed Test for total coliforms. See Figure III-B-2. The MPN per 100 ml is calculated from the MPN table based upon the Confirmed or Completed test results.

4.2 Scope and Application

4.2.1 Advantages: The MPN procedure is a tube-dilution method using a nutrient-rich medium, which is less sensitive to toxicity and supports the growth of environmentally-stressed organisms. The method is applicable to the examination of total coliforms in chlorinated primary effluents and under other stressed conditions. The multiple-tube procedure is also better suited for the examination of turbid samples, muds, sediments, or sludges because particulates do not interfere visibly with the test.

4.2.2 Limitations: Certain non-coliform bacteria may suppress coliforms or act synergistically to ferment lauryl tryptose broth and yield false positive results. A significant number of false positive results can also occur in the brilliant green bile broth when chlorinated primary effluents are tested, especially when stormwater is mixed with the sewage (3). False negatives may occur with waters containing nitrates (4). False positives are more common in sediments.

4.3 Apparatus and Materials

4.3.1 Water bath or air incubator set at 35 ± 0.5 C.

4.3.2 Pipet containers of stainless steel, aluminum, or pyrex glass for glass pipets.

4.3.3 Inoculation loops, at least 3 mm diameter and needles of nichrome or platinum wire, 26 B & S gauge, in suitable holders.

4.3.4 Disposable sterile applicator sticks or plastic loops as alternatives to inoculating loops.

4.3.5 Compound microscope, oil immersion.

4.3.6 Bunsen/Fisher burner or electric incinerator unit.

4.3.7 Sterile TD Mohr or bacteriological pipets, glass or plastic, of appropriate size.

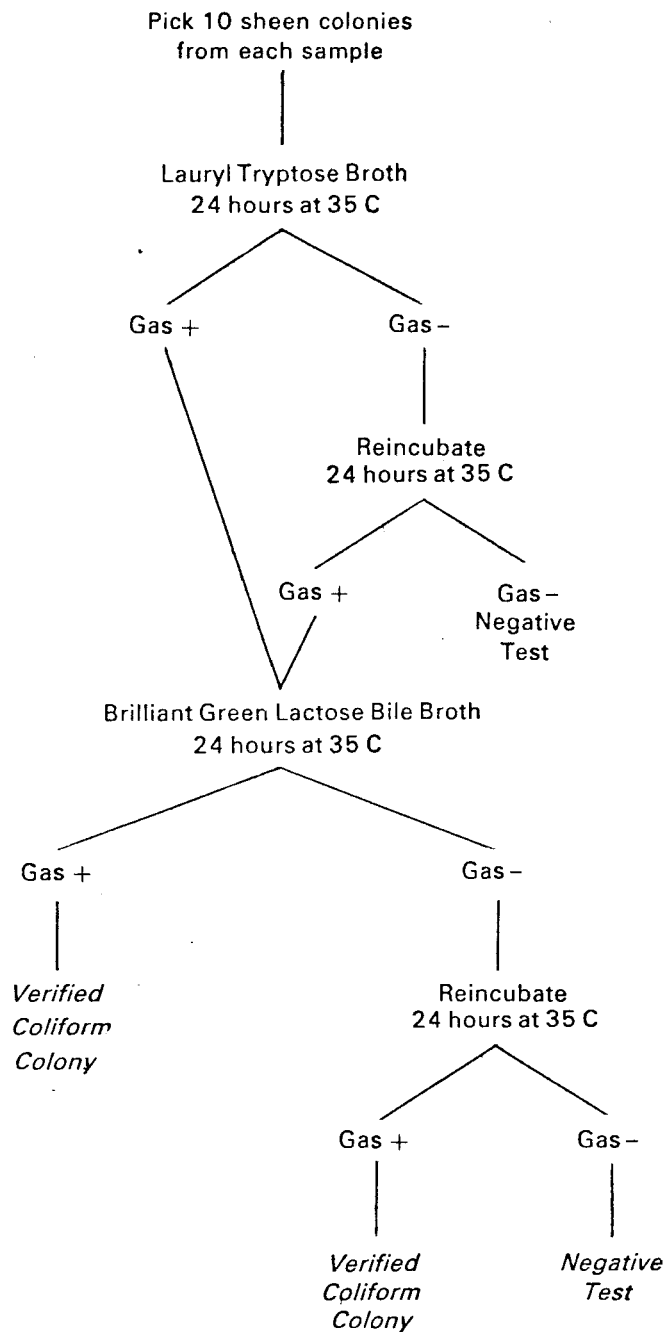


FIGURE III-B-1. Verification of Total Coliform Colonies on the Membrane Filter

TOTAL COLIFORMS

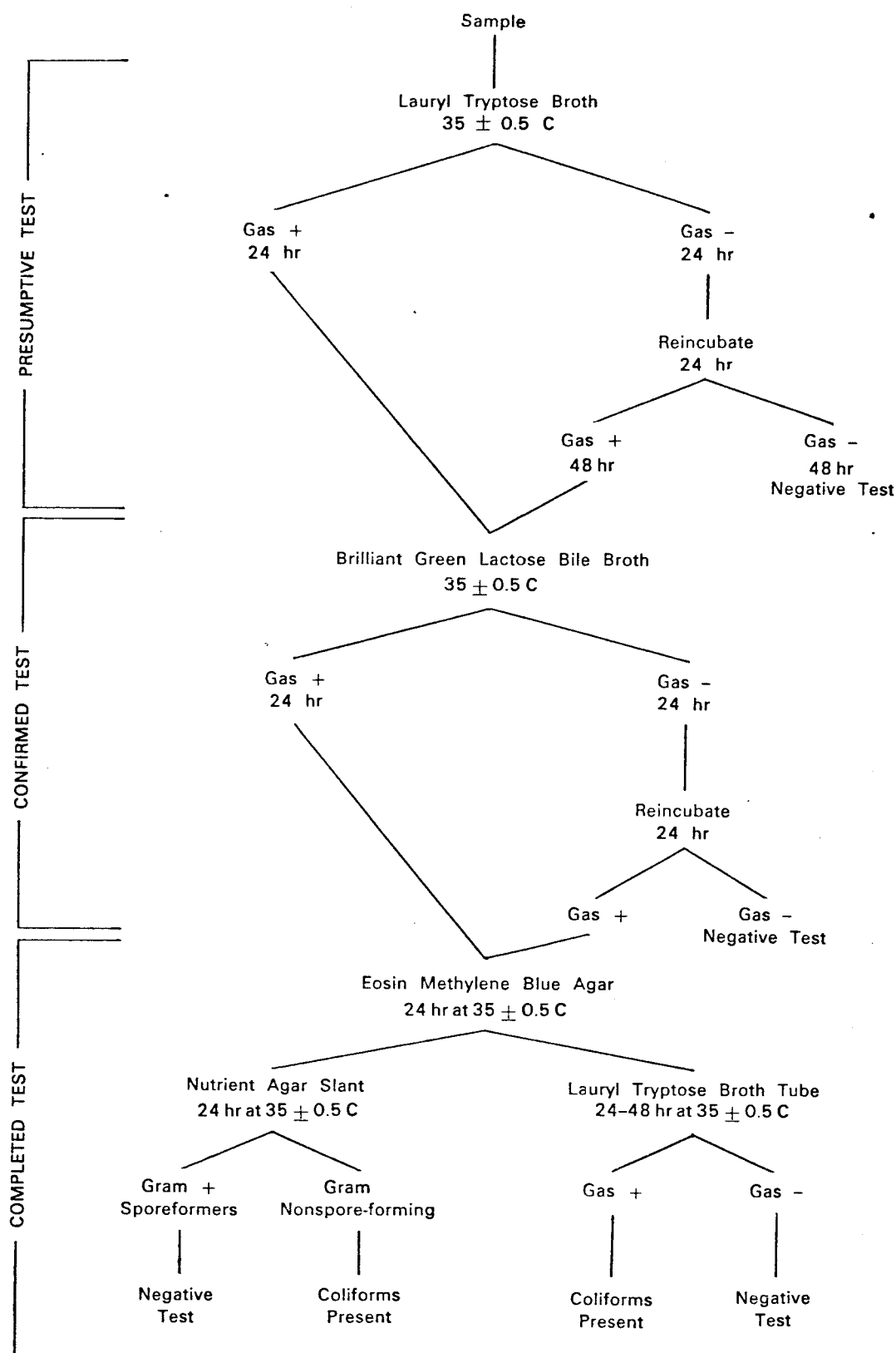


FIGURE III-B-2. Flow Chart for the Total Coliform MPN Test

4.3.8 Pyrex culture tubes, 150 × 25 mm or 150 × 20 mm, containing inverted fermentation vials, 75 × 10 mm with caps.

4.3.9 Culture tube racks to hold fifty, 25 mm diameter tubes.

4.3.10 Dilution bottles (milk dilution) pyrex glass, 99 ml volume, screw cap with neoprene rubber liners.

4.4 Media

4.4.1 Presumptive Test: Lauryl tryptose broth. See Part II-B, 5.3.1. Lactose broth is not used because of false positive reactions.

4.4.2 Confirmed Test: Brilliant green bile broth. (See Part II-B, 5.3.2).

4.4.3 Completed Test:

(a) Eosin methylene blue agar (see Part II-B, 5.3.3).

(b) Nutrient agar or plate count agar slants (see Part II-B, 5.1.1 and 5.1.5).

4.5 Dilution Water: Sterile dilution water dispensed in 99 ± 2 ml amounts preferably in screw-capped bottles. (See Part II-B, 7).

4.6 Procedure: Part II-C describes the general MPN procedure in detail.

4.6.1 Prepare the media for Presumptive, Confirmed or Completed Tests selected. (See Part II-B, 5.3).

4.6.2 Presumptive Test (See Figure III-B-2): To begin the Presumptive Test, arrange fermentation tubes of lauryl tryptose broth in rows of 5 tubes each in the tube rack. Select sample volumes and clearly label each bank of tubes to identify the sample and volume inoculated.

(a) For potable waters, five portions of 10 ml each or five portions of 100 ml each are used.

(b) For relatively-unpolluted waters the sample volumes for the five rows might be

100, 10, 1, 0.1 and 0.01 ml, respectively; the latter two volumes delivered as dilutions of original sample.

(c) For known polluted waters the initial sample inoculations might be 0.1, 0.01, 0.001, 0.0001, and 0.00001 ml of original sample delivered as dilutions into successive rows each containing five replicate volumes. This series of sample volumes will yield determinate results from a low of 200 to a high of 16,000,000 organisms per 100 ml.

(d) Shake the sample and dilutions vigorously about 25 times. Inoculate each 5-tube row with replicate sample volumes in increasing decimal dilutions and incubate at 35 C ± 0.5 C.

(e) After 24 ± 2 hours incubation at 35 C, gently agitate the tubes in the rack and examine the tubes for gas. Any amount of gas constitutes a positive test. If there is no gas production in the tubes, reincubate for an additional 24 hours and reexamine for gas. Positive Presumption tubes are submitted directly to the Confirmed Test. Results are recorded on laboratory bench forms.

(f) If a laboratory using the MPN test on water supplies finds frequent numbers of Presumptive test tubes with heavy growth but no gas, these negative tubes should be submitted to the Confirmed Test to check for suppression of coliforms.

(g) If The Presumptive Test tubes are gas-negative after 48 ± 3 hours, they are discarded and the results recorded as negative Presumptive Tests. Positive Presumptive tubes are verified by the Confirmed Test.

(h) If the fecal coliform test is to be run, (Part III-C), the analyst can inoculate growth from positive Presumptive Test tubes into EC medium at the same time as he inoculates the Confirmed Test Medium.

4.6.3 Confirmed Test (See Figure III-B-2)

(a) Carefully shake each positive Presumptive tube. With a sterile 3 mm loop or a sterile

applicator stick, transfer growth from each tube to BGLB. Gently agitate the tubes to mix the inoculum and incubate at 35 ± 0.5 C.

(b) After 24 ± 2 hours incubation at 35 C examine the tubes for gas. Any amount of gas in BGLB constitutes a positive Confirmed Test. If there is no gas production in the tubes (negative test) reincubate tubes for an additional 24 hours. Record the gas-positive and gas-negative tubes. Hold the positive tubes for the Completed Test if required for quality control or for checks on questionable reactions.

(c) After 48 ± 3 hours reexamine the Confirmed Test Tubes. Record the positive and negative tube results. Discard the negative tubes and hold the positive tubes for the Completed Test if required as in (b) above.

(d) In routine practice most sample analyses are terminated at the end of the Confirmed Test. However, the Confirmed Test data should be verified by carrying 5% of Confirmed Tests with a minimum of one sample per test run through the Completed Test.

(e) For certification of water supply laboratories, the MPN test is carried to completion (except for gram stain) on 10 percent of positive confirmed samples and at least one sample quarterly.

4.6.4 Completed Test (See Figure III-B-2)

Positive Confirmed Test cultures may be subjected to final Completed Test identification through application of further biochemical and culture tests, as follows:

(a) Streak one or more EMB agar plates from each positive BGLB tube. Incubate the plates at 35 ± 0.5 C for 24 ± 2 hours.

(b) Transfer one or more well-isolated typical colonies (nucleated with or without a metallic sheen) to lauryl tryptose broth fermentation tubes and to nutrient or plate count agar slants. Incubate the slants for 24 ± 2 or 48 ± 3 hours at 35 ± 0.5 C. If no typical colonies are present, pick and inoculate at least two atypical (pink, mucoid and unnucleated) colonies

into lauryl tryptose fermentation tubes and incubate tubes for up to 48 ± 3 hours.

(c) The formation of gas in any amount in the fermentation tubes and presence of gram negative rods constitute a positive Completed Test for total coliforms.

4.6.5 Special Considerations for Potable Waters

Sample Size - For potable waters the standard sample shall be five times the standard portion which is either 10 milliliters or 100 milliliters as described in 40 CFR 141 (5).

Confirmation - If a laboratory using the MPN test on water supplies finds frequent numbers of Presumptive test tubes with heavy growth but no gas, these negative tubes should be submitted to the Confirmed Test to check for suppression of coliforms.

Completion - In water supply laboratories, 10% of all samples and at least one sample quarterly must be carried to completion but no gram stain of cultures is required.

4.7 Calculations: The results of the Confirmed or Completed Test may be obtained from the MPN table based on the number of positive tubes in each dilution. See Part II-C, 4.9 for details on calculation of MPN results.

4.7.1 Table II-C-4 illustrates the MPN index and 95% Confidence Limits for combinations of positive and negative results when five 10 ml, five 1.0 ml, and five 0.1 ml volumes of sample are tested.

4.7.2 Table II-C-5 provides the MPN indices and limits for the five tube, single volumes used for potable water supplies.

4.7.3 When the series of decimal dilutions is other than those in the tables select the MPN value from Table II-C-4 and calculate according to the following formula:

$$\text{MPN (From Table)} \times \frac{10}{\text{Largest Volume Tested}} \\ = \text{MPN/100 ml}$$

4.8 Reporting Results: Report the MPN values per 100 ml of sample. See an example of a report form in Figures III-D-2 and III-D-3.

4.9 Precision and Accuracy: The precision of the MPN value increases with increased numbers of replicates tested. A five tube, five dilution MPN is recommended for natural and waste waters. Only a five tube, single volume series is required for potable waters.

5. Differentiation of the Coliform Group by Further Biochemical Tests

5.1 Summary: The differentiation of the members of the coliform group into genera and species is based on additional biochemical and cultural tests (see Table III-B-1). These tests require specific training for valid results.

5.2 Apparatus and Materials

5.2.1 Incubator set at 35 ± 0.5 C.

5.2.2 Pipet containers of stainless steel, aluminum or pyrex glass for glass pipets.

5.2.3 Inoculation loop, 3 mm diameter and needle.

5.2.4 Bunsen/Fisher type burner or electric incinerator.

5.2.5 Sterile TD Mohr and bacteriological pipets, glass or plastic, of appropriate volumes.

5.2.6 Graduates, 25 - 500 ml.

5.2.7 Test tubes, 100×13 mm or 150×20 mm with caps, in racks.

5.2.8 Reagents

(a) **Indole Test Reagent:** Dissolve 5 grams para-dimethylamino benzaldehyde in 75 ml isoamyl (or normal amyl) alcohol, ACS grade, and slowly add 25 ml conc HCl. The reagent should be yellow and have a pH below 6.0. If the final reagent is dark in color it should be discarded.

Some brands are not satisfactory and others become unsatisfactory after aging. Both amyl alcohol and benzaldehyde compound should be purchased in as small amounts as will be consistent with the volume of work anticipated. Store the reagent in the dark in a brown bottle with a glass stopper.

(b) **Methyl Red Test Reagent:** Dissolve 0.1 gram methyl red in 300 ml of 95% ethyl alcohol and dilute to 500 ml with distilled water.

(c) Voges-Proskauer Test Reagents

(1) **Naphthol solution:** Dissolve 5 grams purified alphanaphthol (melting point 92.5°C or higher) in 100 ml absolute ethyl alcohol. This solution must be freshly prepared each day.

(2) **Potassium hydroxide solution:** Dissolve 40 grams KOH in 100 ml distilled water.

(d) Oxidase Test Reagents

(1) **Reagent A:** Weigh out 1 gram alphanaphthol and dissolve in 100 ml of 95% ethanol.

(2) **Reagent B:** Weigh out 1 gram para-aminodimethylaniline HCl (or oxybate) and dissolve in 100 ml of distilled water. Prepare frequently and store in refrigerator.

5.3 Media

5.3.1 Tryptophane broth for demonstrating indole production in the Indole Test. (See Part II-B, 5.1.9 (a) for preparation).

5.3.2 MR-VP broth (buffered glucose) to demonstrate acid production by methyl red color change in the Methyl Red Test and to demonstrate acetyl methyl carbinol production in the Voges-Proskauer test. (See Part II-B, 5.1.9 (b) for preparation).

5.3.3 Simmon's Citrate Agar to demonstrate utilization of citrate as a sole source of carbon. (See Part II-B, 5.1.9 (c) for preparation).

TABLE III-B-1

Differentiation of the Coliform and Related Organisms Based
Upon Biochemical Reactions

Bacterium	Tests								Motility
	Indole	Methyl Red	Voges- Proskauer	Citrate	Cytochrome Oxidase	Ornithine Decarboxylase	Lysine Decarboxylase	Arginine Dehydrolase	
<u>Escherichia coli</u>	+	+	-	-	-	V	V	V	±
<u>Citrobacter freundii</u>	-	+	-	+	-	V	V	V	+
<u>Enterobacter aerogenes</u>	-	-	+	+	-	+	+	-	+
<u>Klebsiella</u>	+	-	+	+	-	-	+	-	-
<u>Pseudomonas</u>	-	-	-	+	+	-	-	-(+) ¹	+
<u>Aeromonas</u>	+	-	+	+	+	+	-	+	+

V = variable

()¹ = reaction of *P. aeruginosa*

5.3.4 Nutrient agar slant for oxidase test. (See Part II-B, 5.1.1 for preparation).

5.3.5 Decarboxylase medium base containing lysine HCl, arginine HCl or ornithine HCl to demonstrate utilization of the specific amino acids. (See Part II-B, 5.5.14 for preparation).

5.3.6 Motility test medium (Edwards and Ewing). (See Section II-B, 5.1.10 for preparation).

5.3.7 Multitest Systems (optional to Single Test Series)

- (a) API Enteric 20 (Analytab Products, Inc.).
- (b) Enterotube (Roche Diagnostics).
- (c) Inolex (Inolex Biomedical Division of Wilson Pharmaceutical and Chemical Corp.).
- (d) Minitek (Baltimore Biological Laboratories, Bioquest).
- (e) Pathotec Test Strips (General Diagnostics Division of Warner-Lambert Company).
- (f) r/b Enteric Differential System (Diagnostic Research, Inc.).

5.4 Procedure

5.4.1 Biochemical tests should always be performed along with positive and negative controls. See Table IV-A-5.

5.4.2 Indole Test

(a) Inoculate a pure culture into 5 ml of tryptophane broth.

(b) Incubate the tryptophane broth at 35 ± 0.5 C for 24 ± 2 hours and mix well.

(c) Add 0.2–0.3 ml test reagent to the 24 hour culture, shake and allow the mixture to stand for 10 minutes. Observe and record the results.

(d) A dark red color in the amyl alcohol layer on top of the culture is a positive indole test; the original color of the reagent, a negative test. An orange color may indicate the presence of skatole and is reported as a \pm reaction.

5.4.3 Methyl Red Test

(a) Inoculate a pure culture into 10 ml of buffered glucose broth.

(b) Incubate for 5 days at 35 C.

(c) To 5 ml of the five day culture, add 5 drops of methyl red indicator.

(d) A distinct red color is positive and distinct yellow, negative. Orange color is dubious, may indicate a mixed culture and should be repeated.

5.4.4 Voges Proskauer Test: This procedure detects the production of acetyl methyl carbinol which in the presence of alphanaphthol and potassium hydroxide develops a reddish color.

(a) Use a pure culture to inoculate 10 ml of buffered glucose broth or 5 ml of salt peptone glucose broth or use the previously inoculated buffered glucose broth from the Methyl Red Test.

(b) Incubate the inoculated salt peptone glucose broth or the buffered glucose broth at 35 ± 0.5 C for 48 hours.

(c) Add 0.6 ml naphthol solution and 0.2 ml KOH solution to 1 ml of the 48 hour salt peptone or buffered glucose broth culture in a separate clean test tube. Shake vigorously for 10 seconds and allow the mixture to stand for 2–4 hours.

(d) Observe the results and record. A pink to crimson color is a positive test. Do not read after 4 hours. A negative test may develop a copper or faint brown color.

5.4.5 Citrate Test

(a) Lightly inoculate a pure culture into a tube of Simon's Citrate Agar, using a needle to stab, then streak the medium. Be careful not to carry over any nutrient material.

(b) Incubate at 35 C for 48 hours.

(c) Examine agar tube for growth and color change. A distinct Prussian blue color in the presence of growth indicates a positive test; no color change is a negative test.

5.4.6 Cytochrome Oxidase Test (Indophenol): The cytochrome oxidase test can be done with commercially-prepared paper strips or on a nutrient agar slant as follows:

(a) Inoculate nutrient agar slant and incubate at 35 C for 18–24 hours. Older cultures should not be used.

(b) Add 2–3 drops of reagent A and reagent B to the slant, tilt to mix and read reaction within 2 minutes.

(c) Strong positive reaction (blue color slant or paper strip) occurs in 30 seconds. Ignore weak reactions that occur after 2 minutes.

5.4.7 Decarboxylase Tests (lysine, arginine and ornithine)

(a) The complete decarboxylase test series requires tubes of each of the amino acids and a control tube containing no amino acids.

(b) Inoculate each tube lightly.

(c) Add sufficient sterile mineral oil to the broths to make 3–4 mm layers on the surface and tighten the screw caps.

(d) Incubate for 18–24 hours at 35 C and read. Negative reactions should be re-incubated up to 4 days.

(e) Positive reactions are purple and negative reactions are yellow. Read the control tube without amino acid first; it must be yellow

for the reactions of the other tubes to be valid. Positive purple tubes must have growth as evidenced by turbidity because uninoculated tubes are also purple; nonfermenters may remain alkaline throughout incubation.

5.4.8 Motility Test

(a) Stab-inoculate the center of the tube of Motility Test Medium to at least half depth.

(b) Incubate tubes 24–48 hours at 35 C.

(c) Examine tubes for growth. If negative, reincubate at room temperature for 5 more days.

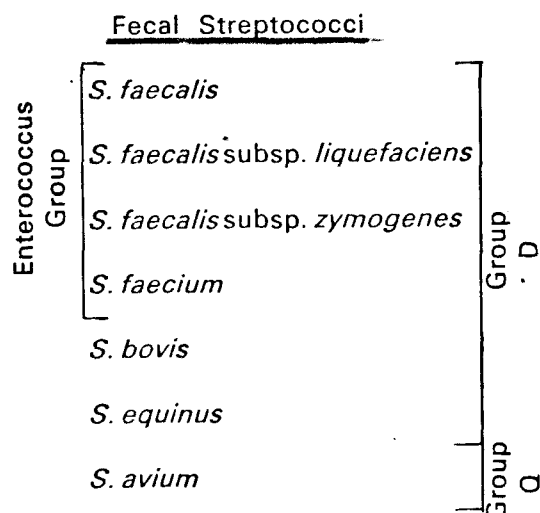
(d) Non-motile organisms grow only along the line of inoculation. Motile organisms grow outward from the line of inoculation and spread throughout the medium producing a cloudy appearance.

(e) Addition of 2, 3, 5 triphenyl tetrazolium chloride (TTC) will aid recognition of motility. Growth of microorganisms reduces TTC and produces red color along the line of growth.

5.4.9 Additional Biochemical Tests: If other biochemical tests are necessary to further identify enteric bacteria, for example specific carbohydrate fermentation, see the Table III-E-5, *Biochemical Characteristics of Enterobacteriaceae*.

5.4.10 Multitest Systems: Multitest systems are available which use tubes containing agar media that provide numerous biochemical tests, plastic units containing a series of dehydrated media, media-impregnated discs and reagent-impregnated paper strips. Some of the systems use numerical codes to aid identification. Others provide computerized identification of bacteria. A number of independent investigators have compared one or more multitest systems with conventional or traditional biochemical tests. Some of the earlier systems have been improved. Most of the recent studies report the correct identification of high percentages of isolates. The systems are described in Part III-E, 5.6.

The fecal streptococci group includes the serological groups D and Q.



1.4 Viridans Streptococci: The viridans streptococci, primarily *S. salivarius* and *S. mitis* are not considered as part of the fecal streptococci as defined in 1.2 and 1.3. These inhabitants of the nasopharyngeal tract have been reported by a few workers in feces and do grow on some fecal streptococci media. However, their low numbers when present, the low frequency of occurrence and the limited data available at this time concerning their presence, have resulted in their exclusion from the classification of fecal streptococci.

1.5 Scope and Application: Fecal streptococci data verify fecal pollution and may provide additional information concerning the recency and probable origin of pollution. In combination with data on coliform bacteria, fecal streptococci are used in sanitary evaluation as a supplement to fecal coliforms when a more precise determination of sources of contamination is necessary. The occurrence of fecal streptococci in water indicates fecal contamination by warm-blooded animals. They are not known to multiply in the environment. Further identification of streptococcal types present in the sample may be obtained by biochemical characterization. (See Figure III-D-2 "Isolation and Identification of Fecal Streptococci"). Such information is useful for source investigations. For example, *S. bovis* and *S. equinus* are host specific and are associated with the fecal excrement of non-human warm-blooded

animals. High numbers of these organisms are associated with pollution from meat processing plants, dairy wastes, and run-off from feedlots and farmlands. Because of limited survival time outside the animal intestinal tract their presence indicates very recent contamination from farm animals.

2. Membrane Filter (MF) Method

2.1 Summary: A suitable volume of sample is passed through the 0.45 μ m membrane filter which retains the bacteria. The filter is placed on KF Streptococcus agar and incubated at 35 C for 48 hours. Red and pink colonies are counted as streptococci (1, 2).

2.2 Scope and Application: The membrane filter technique is recommended as the standard method for assaying fecal streptococci in fresh and marine waters and in non-chlorinated sewage. Wastewaters from food processing plants, slaughter houses, canneries, sugar processing plants, dairy plants, feedlot and farmland run-off may be analyzed by this procedure. Colonies on a membrane filter can be transferred to biochemical media for identification and speciation to provide information on the source of contamination. The general advantages and limitations of the MF method are given in Part II-C.

2.3 Apparatus and Materials

2.3.1 Water bath, aluminum heat sink or air incubator set at 35 ± 0.5 C. Temperature checked with an NBS thermometer or one of equivalent accuracy.

2.3.2 Stereoscopic (dissection) microscope, with magnification of 10 to 15 \times , preferably wide field type. A microscope lamp with diffuse light from cool, white fluorescent tubes is recommended.

2.3.3 Hand tally.

2.3.4 Pipet containers of stainless steel, aluminum or pyrex glass for glass pipets.

2.3.5 Sterile graduated cylinders, covered with aluminum foil or kraft paper.

2.3.6 Sterile, unassembled membrane filtration units (filter base and funnel), glass, plastic or stainless steel, wrapped with aluminum foil or kraft paper.

2.3.7 Vacuum source.

2.3.8 Filter suction flask to hold filter base, with appropriate tubing. Filter manifolds that hold a number of filter bases can also be used.

2.3.9 Safety vacuum flask.

2.3.10 Forceps, with smooth tips.

2.3.11 Ethanol, 95%, or methanol, in small vial for sterilizing forceps.

2.3.12 Bunsen/Fisher burner or electric incinerator.

2.3.13 Sterile T.D. bacteriological or Mohr pipets, glass or plastic, of appropriate size.

2.3.14 Sterile petri dishes, 50 × 12 mm plastic or 60 × 15 mm glass or plastic.

2.3.15 Dilution bottles (milk dilution), pyrex glass, marked at 99 ml volumes, screw-cap with neoprene rubber liner.

2.3.16 Membrane filters, manufactured from cellulose ester materials, white, grid marked, 47 mm in diameter, $0.45 \pm 0.02 \mu\text{m}$ pore size or other pore size as recommended by the manufacturer for fecal streptococci analyses.

2.3.17 Ultraviolet sterilizer for MF filtration units (optional).

2.4 Media: KF Streptococcus agar prepared as described in Part II-B, 5.4.1.

2.5 Dilution Water

2.5.1 Sterile buffered dilution water or peptone water dispensed in 99 ± 2 ml volumes in screw-capped dilution bottles.

2.5.2 Sterile buffered water or peptone water prepared as described in Part II-B, 7, in large volumes for wetting membranes before the addition of the sample, and for rinsing the funnel after filtration.

2.6 Immediate MF Procedure: The general membrane filter procedure is described in detail in Part II-C, 3.

2.6.1 Clearly mark each petri dish and aseptically add 5–6 ml of the liquified agar medium (to each dish to a depth of 2–3 mm).

2.6.2 Place a sterile membrane filter on the filter base, grid-side up and attach the funnel to the base of the filter unit; the membrane filter is now held securely between the funnel and base.

2.6.3 Shake the sample vigorously about 25 times and measure the sample into the funnel with the vacuum off.

2.6.4 Filter appropriate volumes of water sample through the sterile membrane to obtain 20–100 colonies on the membrane surface.

At least 3 sample increments should be filtered in order of increasing volumes. Where no background information is available, more may be necessary. The methods of measurement and dispensation of the sample into the funnel are given in Part II-C, 3.4.6.

2.6.5 Filter the sample and rinse the sides of the funnel at least twice with 20–30 ml of sterile buffered dilution water. Turn off the vacuum and remove the funnel from the filter base.

2.6.6 Aseptically remove the membrane from the filter base and place grid-side up on the agar.

2.6.7 Incubate the petri dishes in the inverted position at 35 ± 0.5 C for 48 hours.

2.6.8 After incubation, remove dishes and examine for red to pink colonies and count.

2.7 Counting and Recording Colonies: Select those plates with 20-100 pink to dark red colonies. These may range in size from barely visible to about 2 mm in diameter. Colonies of other colors are not counted. Count the colonies as described in Part II-C, 3.5, using low-power (10-15 \times) microscope equipped with overhead illumination.

Fecal streptococcal density is reported as organisms per 100 ml. Use the general formula to calculate fecal streptococci densities:

$$\begin{array}{l} \text{Fecal Streptococci/100 ml} = \\ \frac{\text{No. of Fecal Streptococcus Colonies Counted}}{\text{Volume of Sample Filtered, ml}} \\ \times 100 \end{array}$$

For example, if 40 colonies are counted after the filtration of 50 ml of sample the calculation is:

$$\text{Fecal Streptococci/100 ml} = \frac{40}{50} \times 100 = 80.$$

See Part II-C, 3.6 for calculation for results.

Reporting Results: Report fecal streptococcal densities per 100 ml of sample. See discussion on significant figures in Part II-C, 2.8.

2.8 Precision and Accuracy

2.8.1 Extensive precision and accuracy data are not available, however, KF Streptococcus agar has been reported to be highly efficient in the recovery of fecal streptococci (2, 3, 4). In the analyses of feces, sewage and foods, KF yielded a high recovery of fecal streptococci with a low percent (18.6) of non-fecal streptococci.

2.8.2 Laboratory personnel should be able to duplicate their colony counts on the same plates within 5%, and the counts of others within 10%.

2.9 Delayed MF Procedure: Because of the stability of the KF agar and its extreme selectivity for fecal streptococci, it is possible

to filter water samples at a field site, place membranes on the KF agar medium in tight-lidded petri dishes and hold these plates for up to 3 days. After the holding period, plates are incubated for 48 hours at 35 C and counted in the normal manner. This 72 hour holding time can be used to air mail the membranes on KF agar to a central laboratory for incubation and counting. (National Pollution Surveillance System FWPCA, data collected from geographical locations around the Nation; and Kenner *et al.*, Kansas City data.)

3. Verification

Periodically, typical colonies growing on the membrane filter should be verified. When a survey is initiated or a new body of water is being sampled, it is recommended that at least 10 typical colonies from the membrane or agar plate used in computing the final density be picked and transferred into BHI broth or onto BHI agar slants. After 24-48 hours incubation, subject the cultures to a catalase test. Catalase activity indicates the nonstreptococci. Atypical colonies should also be verified to determine false negative reactions on the membrane filter. Final confirmation of fecal streptococci is achieved by determining growth of catalase negative isolates in BHI broth at 45 C and in 40% bile within two days (see Figure III-D-1).

3.1 Apparatus and Materials

3.1.1 Incubators set at 35 ± 0.5 C and 45 ± 0.5 C.

3.1.2 Inoculating needle and loop.

3.1.3 Bunsen/Fisher burner or electric incinerator.

3.1.4 Solution of 3% hydrogen peroxide.

3.1.5 Glass microscope slides, 2.5 \times 7.6 cm (1 \times 3 inches).

TURBIDITY

Method 180.1 (Nephelometric)

STORET NO. 00076

1. Scope and Application
 - 1.1 This method is applicable to drinking, surface, and saline waters in the range of turbidity from 0 to 40 nephelometric turbidity units (NTU). Higher values may be obtained with dilution of the sample.

NOTE 1: NTU's are considered comparable to the previously reported Formazin Turbidity Units (FTU) and Jackson Turbidity Units (JTU).
2. Summary of Method
 - 2.1 The method is based upon a comparison of the intensity of light scattered by the sample under defined conditions with the intensity of light scattered by a standard reference suspension. The higher the intensity of scattered light, the higher the turbidity. Readings, in NTU's, are made in a nephelometer designed according to specifications outlined in Apparatus. A standard suspension of Formazin, prepared under closely defined conditions, is used to calibrate the instrument.
 - 2.1.1 Formazin polymer is used as the turbidity reference suspension for water because it is more reproducible than other types of standards previously used for turbidity standards.
 - 2.1.2 A commercially available polymer standard is also approved for use for the National Interim Primary Drinking Water Regulations. This standard is identified as AMCO-AEPA-1 available from Amco Standard International, Inc.
3. Sample Handling and Preservation
 - 3.1 Preservation of the sample is not practical; analysis should begin as soon as possible. Refrigeration or icing to 4°C, to minimize microbiological decomposition of solids, is recommended.
4. Interferences
 - 4.1 The presence of floating debris and coarse sediments which settle out rapidly will give low readings. Finely divided air bubbles will affect the results in a positive manner.
 - 4.2 The presence of true color, that is the color of water which is due to dissolved substances which absorb light, will cause turbidities to be low, although this effect is generally not significant with finished waters.
5. Apparatus
 - 5.1 The turbidimeter shall consist of a nephelometer with light source for illuminating the sample and one or more photo-electric detectors with a readout device to indicate the intensity of light scattered at right angles to the path of the incident light. The turbidimeter should be so designed that little stray light reaches the detector in the

Approved for NPDES and SDWA
Issued 1971
Editorial revision 1974
Editorial revision 1978

absence of turbidity and should be free from significant drift after a short warm-up period.

- 5.2 The sensitivity of the instrument should permit detection of a turbidity difference of 0.02 unit or less in waters having turbidities less than 1 unit. The instrument should measure from 0 to 40 units turbidity. Several ranges will be necessary to obtain both adequate coverage and sufficient sensitivity for low turbidities.
- 5.3 The sample tubes to be used with the available instrument must be of clear, colorless glass. They should be kept scrupulously clean, both inside and out, and discarded when they become scratched or etched. They must not be handled at all where the light strikes them, but should be provided with sufficient extra length, or with a protective case, so that they may be handled.
- 5.4 Differences in physical design of turbidimeters will cause differences in measured values for turbidity even though the same suspension is used for calibration. To minimize such differences, the following design criteria should be observed:
 - 5.4.1 Light source: Tungsten lamp operated at a color temperature between 2200–3000°K.
 - 5.4.2 Distance traversed by incident light and scattered light within the sample tube: Total not to exceed 10 cm.
 - 5.4.3 Detector: Centered at 90° to the incident light path and not to exceed $\pm 30^\circ$ from 90°. The Detector, and filter system if used, shall have a spectral peak response between 400 and 600nm.
- 5.5 The Hach Turbidimeter, Model 2100 and 2100 A, is in wide use and has been found to be reliable; however, other instruments meeting the above design criteria are acceptable.

6. Reagents

- 6.1 Turbidity-free water: Pass distilled water through a 0.45 μ pore size membrane filter if such filtered water shows a lower turbidity than the distilled water.
- 6.2 Stock formazin turbidity suspension:

Solution 1: Dissolve 1.00 g hydrazine sulfate, $(\text{NH}_2)_2\cdot\text{H}_2\text{SO}_4$, in distilled water and dilute to 100 ml in a volumetric flask.

Solution 2: Dissolve 10.00 g hexamethylenetetramine in distilled water and dilute to 100 ml in a volumetric flask.

In a 100 ml volumetric flask, mix 5.0 ml Solution 1 with 5.0 ml Solution 2. Allow to stand 24 hours at $25 \pm 3^\circ\text{C}$, then dilute to the mark and mix.
- 6.3 Standard formazin turbidity suspension: Dilute 10.00 ml stock turbidity suspension to 100 ml with turbidity-free water. The turbidity of this suspension is defined as 40 units. Dilute portions of the standard turbidity suspension with turbidity-free water as required.
 - 6.3.1 A new stock turbidity suspension should be prepared each month. The standard turbidity suspension and dilute turbidity standards should be prepared weekly by dilution of the stock turbidity suspension.
- 6.4 The AMCO-AEPA-1 standard as supplied requires no preparation or dilution prior to use.

7. Procedure

7.1 Turbidimeter calibration: The manufacturer's operating instructions should be followed. Measure standards on the turbidimeter covering the range of interest. If the instrument is already calibrated in standard turbidity units, this procedure will check the accuracy of the calibration scales. At least one standard should be run in each instrument range to be used. Some instruments permit adjustments of sensitivity so that scale values will correspond to turbidities. Reliance on a manufacturer's solid scattering standard for setting overall instrument sensitivity for all ranges is not an acceptable practice unless the turbidimeter has been shown to be free of drift on all ranges. If a pre-calibrated scale is not supplied, then calibration curves should be prepared for each range of the instrument.

7.2 Turbidities less than 40 units: Shake the sample to thoroughly disperse the solids. Wait until air bubbles disappear then pour the sample into the turbidimeter tube. Read the turbidity directly from the instrument scale or from the appropriate calibration curve.

7.3 Turbidities exceeding 40 units: Dilute the sample with one or more volumes of turbidity-free water until the turbidity falls below 40 units. The turbidity of the original sample is then computed from the turbidity of the diluted sample and the dilution factor. For example, if 5 volumes of turbidity-free water were added to 1 volume of sample, and the diluted sample showed a turbidity of 30 units, then the turbidity of the original sample was 180 units.

7.3.1 The Hach Turbidimeters, Models 2100 and 2100A, are equipped with 5 separate scales: 0-0.2, 0-1.0, 0-100, and 0-1000 NTU. The upper scales are to be used only as indicators of required dilution volumes to reduce readings to less than 40 NTU.

NOTE 2: Comparative work performed in the MDQAR Laboratory indicates a progressive error on sample turbidities in excess of 40 units.

8. Calculation

8.1 Multiply sample readings by appropriate dilution to obtain final reading.

8.2 Report results as follows:

<u>NTU</u>	<u>Record to Nearest:</u>
0.0 - 1.0	0.05
1 - 10	0.1
10 - 40	1
40 - 100	5
100 - 400	10
400 - 1000	50
> 1000	100

9. Precision and Accuracy

9.1 In a single laboratory (EMSL), using surface water samples at levels of 26, 41, 75 and 180 NTU, the standard deviations were ± 0.60 , ± 0.94 , ± 1.2 and ± 4.7 units, respectively.

9.2 Accuracy data are not available at this time.

Bibliography

1. Annual Book of ASTM Standards, Part 31, "Water", Standard D1889-71, p 223 (1976).
2. Standard Methods for the Examination of Water and Wastewater, 14th Edition, p 132, Method 214A, (1975).

CHLORIDE

MOHR METHOD For Water

This is the most widely used method for chloride. A stable, combined indicator-buffer formulation is used in the following procedure. It adjusts the pH of the sample automatically.

A Standard Sodium Chloride Solution is available to test the accuracy of the test.

Procedure

1. Measure a 100 ml (Note A) water sample and pour it into a 250 ml flask.
2. Add the contents of one Chloride Indicator Powder Pillow. Swirl to mix.
3. Titrate with Standard Silver Nitrate until a permanent shade of red (Note B) appears.
4. Read the buret, and subtract the reagent blank from the buret reading (see Note B). Multiply the remainder by 5 to obtain the ppm chloride in the water sample.

For Wastewater

Chloride is one of the main ions in sewage because salt (sodium chloride) is basic in the diet and passes through the digestive system unchanged. The wide use of zeolite water softeners adds a great deal of chloride to the sewage system.

The most common method for the determination of chloride is the Mohr Method, in which a sample is titrated with a Standard Silver Nitrate Solution using chromate as the end-point indicator. A color change from yellow to permanent red occurs. Sulfide, if present in the sample, also titrates as chloride and will be included in the chloride titration. Sulfide may be removed readily as directed in the procedure.

Procedure

Step 1 is used when sulfide is present, but may be omitted if sulfide is known to be absent.

1. Take approximately 125 ml of sample and add contents of one Lead Carbonate Powder Pillow. Swirl to mix for a minute. Filter through folded filter paper.
2. Measure 100 ml (Note A) of filtered water sample prepared in step one above or 100 ml of sulfide free sample and pour it into a flask or beaker.
3. Add the contents of one powder pillow of Chloride Indicator Powder. Swirl to mix.
4. Titrate with standard Silver Nitrate until a permanent shade of red appears. (Note B)
5. Read the buret, and subtract the reagent blank from the buret reading (Note B). Multiply the difference by 5 to obtain the ppm chloride in the sample.

Reagents and Apparatus

Standard Silver Nitrate, 0.0141N, APHA, No. 316	quart	\$ 2.20
Chloride Indicator Powder Pillows, No. 1057	100	2.95
Lead Carbonate Powder Pillows, No. 1808	100	3.50
Cylinder, Graduated, 250 ml, No. 508	each	5.08
Buret, Automatic, 25 ml, No. 504	each	12.15
Flask, Erlenmeyer, 250 ml, No. 505	each	.53
Clippers for opening Pillows, No. 968	each	.69

Notes for Chloride

A. In most cases, a 100 ml water sample is appropriate. If however, the chloride content is abnormally high, such that the volume of silver nitrate used to titrate 100 ml is above 25 ml, it is advisable to repeat the test, using a smaller sample diluted to 100 ml with demineralized water. The appropriate correction must be made in the calculation. For example, if a 25 ml water sample was taken and diluted to 100 ml with demineralized water, multiply the ml of silver nitrate used by 20 to obtain the ppm chloride in the water sample.

B. The end-point is the point where the first definite red-brown shade of color appears. It is wise to practice a few times using demineralized water as the sample. By doing this, one can ob-

APPENDIX B: Non-Potable Water Tests

- (i) Oil and grease analysis
- (ii) Settleable solids (residue, settleable matter) analysis

OIL AND GREASE, TOTAL, RECOVERABLE

Method 413.1 (Gravimetric, Separatory Funnel Extraction)

STORET NO. 00556

1. Scope and Application
 - 1.1 This method includes the measurement of fluorocarbon-113 extractable matter from surface and saline waters, industrial and domestic wastes. It is applicable to the determination of relatively non-volatile hydrocarbons, vegetable oils, animal fats, waxes, soaps, greases and related matter.
 - 1.2 The method is not applicable to measurement of light hydrocarbons that volatilize at temperatures below 70°C. Petroleum fuels from gasoline through #2 fuel oils are completely or partially lost in the solvent removal operation.
 - 1.3 Some crude oils and heavy fuel oils contain a significant percentage of residue-type materials that are not soluble in fluorocarbon-113. Accordingly, recoveries of these materials will be low.
 - 1.4 The method covers the range from 5 to 1000 mg/l of extractable material.
2. Summary of Method
 - 2.1 The sample is acidified to a low pH (< 2) and serially extracted with fluorocarbon-113 in a separatory funnel. The solvent is evaporated from the extract and the residue weighed.
3. Definitions
 - 3.1 The definition of oil and grease is based on the procedure used. The nature of the oil and/or grease, and the presence of extractable non-oily matter will influence the material measured and interpretation of results.
4. Sampling and Storage
 - 4.1 A representative sample of 1 liter volume should be collected in a glass bottle. If analysis is to be delayed for more than a few hours, the sample is preserved by the addition of 5 ml HCl (6.1) at the time of collection and refrigerated at 4°C.
 - 4.2 Because losses of grease will occur on sampling equipment, the collection of a composite sample is impractical. Individual portions collected at prescribed time intervals must be analyzed separately to obtain the average concentration over an extended period.
5. Apparatus
 - 5.1 Separatory funnel, 2000 ml, with Teflon stopcock.
 - 5.2 Vacuum pump, or other source of vacuum.
 - 5.3 Flask, boiling, 125 ml (Corning No. 4100 or equivalent).
 - 5.4 Distilling head, Claisen or equivalent.
 - 5.5 Filter paper, Whatman No. 40, 11 cm.
6. Reagents
 - 6.1 Hydrochloric acid, 1:1. Mix equal volumes of conc. HCl and distilled water.

Approved for NPDES

Issued 1974

Editorial revision 1978

6.2 Fluorocarbon-113, (1,1,2-trichloro-1,2,2-trifluoroethane), b. p. 48°C.

6.3 Sodium sulfate, anhydrous crystal.

7. Procedure

7.1 Mark the sample bottle at the water meniscus for later determination of sample volume. If the sample was not acidified at time of collection, add 5 ml hydrochloric acid (6.1) to the sample bottle. After mixing the sample, check the pH by touching pH-sensitive paper to the cap to insure that the pH is 2 or lower. Add more acid if necessary.

7.2 Pour the sample into a separatory funnel.

7.3 Tare a boiling flask (pre-dried in an oven at 103°C and stored in a desiccator).

7.4 Add 30 ml fluorocarbon-113 (6.2) to the sample bottle and rotate the bottle to rinse the sides. Transfer the solvent into the separatory funnel. Extract by shaking vigorously for 2 minutes. Allow the layers to separate, and filter the solvent layer into the flask through a funnel containing solvent moistened filter paper.

NOTE: An emulsion that fails to dissipate can be broken by pouring about 1 g sodium sulfate (6.3) into the filter paper cone and slowly draining the emulsion through the salt. Additional 1 g portions can be added to the cone as required.

7.5 Repeat (7.4) twice more, with additional portions of fresh solvent, combining all solvent in the boiling flask.

7.6 Rinse the tip of the separatory funnel, the filter paper, and then the funnel with a total of 10–20 ml solvent and collect the rinsings in the flask.

7.7 Connect the boiling flask to the distilling head and evaporate the solvent by immersing the lower half of the flask in water at 70°C. Collect the solvent for reuse. A solvent blank should accompany each set of samples.

7.8 When the temperature in the distilling head reaches 50°C or the flask appears dry remove the distilling head. Sweep out the flask for 15 seconds with air to remove solvent vapor by inserting a glass tube connected to a vacuum source. Immediately remove the flask from the heat source and wipe the outside to remove excess moisture and fingerprints.

7.9 Cool the boiling flask in a desiccator for 30 minutes and weigh.

8. Calculation

8.1 $\text{mg/l total oil and grease} = \frac{R - B}{V}$

where:

R = residue, gross weight of extraction flask minus the tare weight, in milligrams.

B = blank determination, residue of equivalent volume of extraction solvent, in milligrams.

V = volume of sample, determined by refilling sample bottle to calibration line and correcting for acid addition if necessary, in liters.

9. Precision and Accuracy

- 9.1 The two oil and grease methods in this manual were tested by a single laboratory (EMSL) on sewage. This method determined the oil and grease level in the sewage to be 12.6 mg/l. When 1 liter portions of the sewage were dosed with 14.0 mg of a mixture of #2 fuel oil and Wesson oil, the recovery was 93% with a standard deviation of ± 0.9 mg/l.

Bibliography

1. Standard Methods for the Examination of Water and Wastewater, 14th Edition, p 515, Method 502A, (1975).
2. Blum, K. A., and Taras, M. J., "Determination of Emulsifying Oil in Industrial Wastewater", JWPCF Research Suppl. 40, R404 (1968).

SETTLEABLE MATTER

Method 160.5 (Volumetric, Imhoff Cone)

STORET NO. 50086

1. Scope and Application
 - 1.1 This method is applicable to surface and saline waters, domestic and industrial wastes.
 - 1.2 The practical lower limit of the determination is about 1 ml/1/hr.
2. Summary of Method
 - 2.1 Settleable matter is measured volumetrically with an Imhoff cone.
3. Comments
 - 3.1 For some samples, a separation of settleable and floating materials will occur; in such cases the floating materials are not measured.
 - 3.2 Many treatment plants, especially plants equipped to perform gravimetric measurements, determine residue non-filterable (suspended solids), in preference to settleable matter, to insure that floating matter is included in the analysis.
4. Precision and Accuracy
 - 4.1 Data on this determination are not available at this time.
5. References
 - 5.1 The procedure to be used for this determination is found in:
Standard Methods for the Examination of Water and Wastewater, 14th Edition, p 95,
Method 208F, Procedure 3a (1975).

208 F. Settleable Matter

1. General Discussion

Settleable matter in surface and saline waters as well as domestic and industrial wastes may be determined and reported on either a volume (milliliters per liter) or a weight (milligrams per liter) basis.

2. Apparatus

The apparatus listed under Sections 208 A.2 and 208 B.2, and an Imhoff cone, are required for a gravimetric test. The volumetric test requires only an Imhoff cone.

3. Procedure

a. By volume: Fill an Imhoff cone to the liter mark with a thoroughly mixed sample. Settle for 45 min, gently stir the sides of the cone with a rod or by spinning, settle 15 min longer, and record the volume of settleable matter in the cone as milliliters per liter. The practical lower limits is about 1 ml/l/hr. Where a separation of settleable and floating materials occurs, do not estimate the floating material.

b. By weight:

1) Determine the suspended matter (in milligrams per liter) in the sample as in Method D, preceding.

2) Pour a well-mixed sample into a glass vessel not less than 9 cm in diameter. Use a sample of not less than 1 l and

sufficient to give a depth of 20 cm. A glass vessel of greater diameter and a larger volume of sample also may be used. Let stand quiescent for 1 hr and, without disturbing the settled or floating material or that which may be floating, siphon 250 ml from the center of the container at a point halfway between the surface of the settled sludge and the liquid surface. Determine the suspended matter (in milligrams per liter) in all or in a portion of this supernatant liquor as directed under Method D. This is the nonsettling matter.

PHYSICAL EXAMINATION

4. Calculation

$$\begin{aligned} &\text{mg/l settleable matter} \\ &= \text{mg/l suspended matter} \\ &\quad - \text{mg/l nonsettling matter} \end{aligned}$$

. APPENDIX C: Miscellaneous

(i) Acid-base titrations for hydrogen peroxide analysis

Quality Control

Assay of Hydrogen Peroxide 50% & 30% H_2O_2

Reagents:

Potassium Permanganate 0.1N (.316%) KMnO_4

Sodium Oxalate Primary Standard Dried $\text{Na}_2\text{C}_2\text{O}_4$

Sulfuric Acid, 6N H_2SO_4

Sulfuric Acid, Conc. H_2SO_4

Procedure:

I. Standardization of MnO_4^- :

1. Weigh out about 0.25 g $\text{Na}_2\text{C}_2\text{O}_4$ (dried) into a 250 ml beaker, record weight, add ca. 100 ml distilled water and add 2 ml conc. H_2SO_4 .
2. Add by buret 20 ml of KMnO_4 soln. and heat beaker to ca. 70°C and start titration. (~37 ml of soln.) to permanent pink end point.

II. Assay of H_2SO_2

1. Accurately weigh 1 ml of H_2O_2 with minimal evaporation and transfer qualitatively to 500 ml volumetric flask, fill to volume, shake.
2. Run a blank and two sample aliquots as follows.
Add 20 ml diluted H_2O_2 into a clean 125 ml flask.
Add 10 ml 6N H_2SO_4 .
3. Titrate with 0.1N KMnO_4 soln. until permanent pink end point.

III. Calculations

- a. Determination of normality of KMnO_4 soln.

$$\text{Normality of } \text{KMnO}_4 = \frac{\text{gm } \text{Na}_2\text{C}_2\text{O}_4}{\text{ml of } \text{KMnO}_4} \times 14.925$$

- b. Determination of % H_2O_2

$$\% \text{H}_2\text{O}_2 = \frac{(\text{ml } \overset{\text{TITRANT}}{\text{sample}} - \text{ml blank}) \times N \text{ KMnO}_4 \times 42.525}{\text{gm of sample}}$$

Stock Label 2
regulation
(+5%)
50.0% \pm 2.5%
30.0% \pm 1.5%